RESTORATION OF WHITEBARK PINE ON A BURN SITE UTILIZING NATIVE ECTOMYCORRHIZAL SUILLOID FUNGI

by

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TABLE OF CONTENTS

1. LITERATURE REVIEW 1

Whitebark Pine 1
Origin and Range 1
Ecology 2
Whitebark Pine and Mycorrhizal Fungi 6
Fire 9
Threats 11
Restoration 15
Seed Source 15
Seedlings 16
Blister Rust Resistance Screening 17
Outplanting 18
Mycorrhizal Fungi 21
Greenhouse Studies Utilizing Suilloid Fungi 22
Nutrient Analysis and Isotopic Patterns 24
Inoculation and Outplanting on Burns 26
GPS and GIS Technology for the Monitoring of Whitebark Pine 31
Restoration of Whitebark Pine in the Beaverhead-Deerlodge National Forest 31
Research Objectives 33
References 35

2. GREENHOUSE STUDY: EFFECT OF ECTOMYCORRHIZAL COLONIZATION ON WHITEBARK PINE SEEDLINGS PLANTED IN SOIL FROM A BURN SITE 52

Introduction 52
Whitebark Pine and Ectomycorrhizal Fungi 54
Suilloid Fungi and Fire 56
Restoration of Whitebark Pine 57
Ectomycorrhizal Fungi in Restoration 59
Effects of Ectomycorrhizal Colonization on Seedlings in a Greenhouse Setting 62
Analyses of Isotopic Signatures for the Investigation of the Plant-Fungal Relationship 64
Research Objectives 66
Materials and Methods 67
Soil Collection and Analysis 67
Seedlings 69

TABLE OF CONTENTS – CONTINUED

Ectomycorrhizal Colonization of Seedlings:
Initial Assessment and Molecular Identification 69
Soil Planting Treatments....71
Final Assessment of Mycorrhizal Colonization....74
Height and Stem Diameter....76
Seedling Biomass....77
Foliar Nutrient and Isotope Assessment 77
Experimental Design....79
Statistical Analysis....80
Results....83
Molecular Analysis of Ectomycorrhizae 83
Assessment of Ectomycorrhizal Colonization....83
Height and Stem Diameter....86
Seedling Biomass....87
Foliar Concentration of Nitrogen and Carbon 93
Foliar Nitrogen Content and Isotope Analysis ($\delta^{15}N$) 93
Foliar Carbon Content and Isotope Analysis ($\delta^{13}C$)....96
Foliar C:N 99
Relation of Foliar Nutrient and Isotope
Status to Number of Ectomycorrhizae....102
Discussion....103
Ectomycorrhizal Colonization of Seedlings 104
Soil Planting Treatments....107
Seedling Parameters....109
Burn Soil 112
Foliar Nutrient Analysis....113
Foliar Nitrogen Isotope Analysis....116
Foliar Carbon Isotope Analysis 120
Summary and Conclusions 122
References....127

3. FIELD STUDY: NATIVE ECTOMYCORRHIZAL FUNGI AND SPATIAL ANALYSES FOR THE RESTORATION OF WHITEBARK PINE ON THE EUREKA BASIN BURN....143

Introduction....143
Whitebark Pine....143
Restoration of Whitebark Pine....146
Whitebark Pine and Ectomycorrhizal Fungi....148
Ectomycorrhizal Fungi in Restoration....150
Monitoring 153

TABLE OF CONTENTS – CONTINUED

The Power of GIS 155
Restoration of Whitebark Pine in the Beaverhead-Deerlodge National Forest 156
Research Objectives 158
Materials and Methods 159
Study Area Description 159
Seedlings 164
Ectomycorrhizal Colonization of Seedlings 165
Planting 165
Seedling Data 167
Monitoring Seedling Health 170
Statistical Analysis 170
Spatial Analyses 171
Slope 172
Topographic Wetness Index 172
Results 175
Seedling Height 175
Seedling Health 175
Ectomycorrhizal Colonization and Seedling Health 176
Spatial Analyses 178
Slope 179
Microsite 182
Topographic Wetness Index 184
Discussion 186
Seedling Survival 187
Ectomycorrhizal Colonization of Seedlings 189
Planting Whitebark Pine on a Burn Site 191
Spatial Analyses 194
Slope and Aspect 194
Microsite 196
Topographic Wetness Index 196
Summary and Conclusions 200
References 202

CUMULATIVE REFERENCES CITED 216

APPENDIX A: Field Study Maps and Seedling Data 244

LIST OF TABLES

Table Page

1.1. Literature for Pine Seedling Responses to Colonization by *Suillus* 25

2.1. Eureka Basin Burn Site Soil Analysis Results.....72

2.2. Soil Planting Treatments for Whitebark Pine Seedlings in the Greenhouse.....74

2.3. Frequency and Abundance of Ectomycorrhizal Colonization of New Root Growth of Whitebark Pine Seedlings.....84

2.4. Average Mean and Standard Deviation for Whitebark Pine Seedling Biomass.....91

2.5. Statistical Significance of Whitebark Pine Seedling Height, Stem Diameter, and Biomass Analyses 92

2.6. Average Mean and Standard Deviation for Foliar Nutrient Content and Isotope Measurements.....100

2.7. Statistical Significance of Foliar Nutrient Content and Isotope Analyses.....101

3.1. Health Status of Whitebark Pine Seedlings by Colonization Status.....177

3.2. Average Slope by Whitebark Pine Seedling Health 181

3.3. Average Slope by Transect.....181

3.4. Whitebark Pine Seedling Mortality by Microsite Type and Position.....183

3.5. Average Mean and Standard Deviation for Topographic Wetness Index by Whitebark Pine Seedling Health 186

LIST OF FIGURES

Figure Page

1.1 Range of Whitebark Pine 2

2.1 Eureka Basin Burn Site 68

2.2 New Root Growth of a Whitebark Pine Seedling in the Greenhouse 75

2.3 *Suillus* Ectomycorrhizae on Whitebark Pine Seedling Root Systems 76

2.4 Foliar Nutrient and Isotope Analysis 78

2.5 Frequency and Abundance of Ectomycorrhizal Colonization of New Root Growth of Whitebark Pine Seedlings 85

2.6 Boxplots and Confidence Interval Plots for Whitebark Pine Seedling Height by Soil Treatment and Colonization Status 87

2.7 Boxplots and Confidence Interval Plots for Whitebark Pine Seedling Biomass by Soil Treatment and Colonization Status 89

2.8 Boxplots and Confidence Interval Plots for Whitebark Pine Seedling New Root Biomass by Soil Treatment and Colonization Status 90

2.9 Boxplots of Foliar Nitrogen Content and Foliar $\delta^{15}N$ of Whitebark Pine Seedlings by Soil Treatment and Colonization Status 94

2.10 Scatterplots of Foliar Nitrogen Content by Foliar $\delta^{15}N$ for All Ttreatment Combinations 95

2.11 Boxplots of Foliar Carbon Content and Foliar $\delta^{13}C$ of Whitebark Pine Seedlings by Soil Treatment and Colonization Status 97

2.12 Scatterplots of Foliar Carbon Content by Foliar $\delta^{13}C$ for All Ttreatment Combinations 98

2.13 Scatterplots of Foliar Nutrient and Isotope Measurements by Soil Treatment and Colonization Status 102

LIST OF FIGURES – CONTINUED

Figure Page

3.1 The Eureka Basin Burn in the Gravelly Moutains of Southwest Montana 160

3.2 The Eureka Basin Burn 2015 Planting Site 161

3.3 Precipitation Accumulation and Soil Moisture for 2015 from the Clover Meadow SNOTEL Station 403 163

3.4 A Whitebark Pine Seedling Planted on the Eureka Basin Burn 166

3.5 The Eureka Burn 2015 Planting Site with Study Transects.....167

3.6 Classification of Microsites 168

3.7 Colonization Status of Whitebark Pine Seedlings on the Eureka Basin Burn 2015 Planting Site.....169

3.8 Methods Flow Chart for the Calculation of Topographic Wetness Index in ArcMap 174

3.9 Boxplot of Whitebark Pine Seedling Height by Colonization Status at Planting.....175

3.10 Overall Health of Whitebark Pine Seedlings for the 2015 and 2016 Monitoring Periods.....176

3.11 Health of Whitebark Pine Seedlings by Colonization Status for the 2015 and 2016 Monitoring Periods 177

3.12 Health of Whitebark Pine Seedlings Across Study Transects 178

3.13 Differences in Field Measured Slope and Slope Extracted from a Spatial Dataset 180

3.14 A Whitebark Pine Seedling Buried in Soil Deposition.....182

3.15 Distribution of Whitebark Pine Seedling Mortality by Microsite Type and Location.....183

LIST OF FIGURES – CONTINUED

Figure Page

3.16 Whitebark Pine Seedling Health and Topographic Wetness Index Across Study Transects 184

3.17 Frequency Distributions of Topographic Wetness Index of Whitebark Pine Seedlings by Transect 185

3.18 Boxplots of Topographic Wetness Index by Health of Whitebark Pine Seedlings 186

CHAPTER ONE

LITERATURE REVIEW

Whitebark Pine

Origin and Range

Whitebark pine, *Pinus albicaulis* Engelm., is a five-needle pine that is crucial to the functioning of high elevation subalpine and treeline ecosystems (Arno and Hoff 1989, Fryer 2002, Ellison et al. 2005, Tomback et al. 2014). This unique keystone species is limited to western North America and lies primarily in two distinct distributions, including a Pacific coastal range from California to British Columbia and a northern Rocky Mountain range from Wyoming to Alberta (Arno and Hoff 1989, Figure 1.1). The high elevation habitat type of whitebark pine often overlaps with designated wilderness area (Cole 1990). Over its range, 49% of existing whitebark occurs in wilderness or national parks and over 95% occurs on federal lands (Keane 2000).

Whitebark pine falls in the subgenus Strobus, the haploxylon or soft pines, along with the Great Basin bristlecone (*P. longaeva* Bailey) and Limber Pine (*P. flexilis* James), and in subsection *Cembrae*, the stone pines, which are characterized by five-needled fascicles and indehiscent cones bearing wingless seeds (Critchfield and Little 1966, Little and Critchfield 1969, Lanner 1990). Whitebark pine is the only stone pine native to North America and only four other species of stone pines exist worldwide including: the Swiss stone pine (*P. cembra* L.), Korean stone pine (*P. koraiensis* Sieb. Et Zucc.), Siberian

stone pine (*P. sibirica* Du Tour), and Japanese stone pine (*P. pumila* Regel) (Lanner 1990, McCaughey and Schmidt 2001).

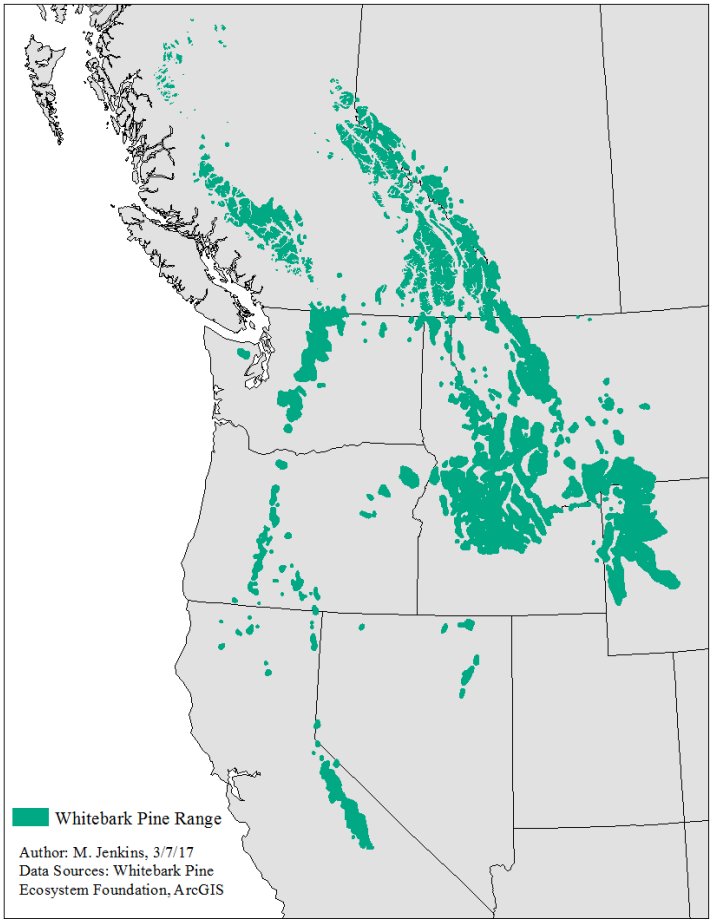

Figure 1.1: Range of whitebark pine (WPEF 2014).

Ecology

Whitebark pine occurs in subalpine, alpine, and treeline ecosystems that are subjected to cold and windy conditions for the majority of the year and in most stands approximately two-thirds of the yearly precipitation comes in the form of snow or sleet (Arno and Hoff 1989). In western Montana, whitebark is found at an elevation range of

5,900 – 9,300 feet. At the highest elevations with the most severe climate conditions, whitebark can occur as a climax species, the single dominant tree species. These individuals often exhibit a krummholz growth form, a low growing horizontally branching form that is the result of harsh windy conditions and a short growing season (Arno and Hoff 1989). One of the important ecological roles of whitebark pine in these high elevation ecosystems is that it can act as an initial colonizer that subsequently facilitates the growth of other less hardy species such as spruce and fir on its leeward (Butler 1986, Callaway 1998). In two paired studies conducted in the northern Rocky Mountains of Montana, krummholz whitebark was found to be the primary tree island initiator at treeline and the most commonly recorded solitary tree species (Resler and Tomback 2008, Tomback et al. 2014). Whitebark pine also occurs as a seral species at lower elevations in mixed Engelmann Spruce-Subalpine Fir habitat in a taller, straighter growth form that is generally more reproductively active due to less harsh climatic conditions (Arno and Hoff 1989, Campbell and Antos 2003). Due to the generally high elevation habitat type, whitebark pine is a slow growing tree that can live for several hundred years; the oldest documented individual, found in the Sawtooth National Forest, ID, exceeds 1,270 years (Perkins and Swetnam 1996).

Whitebark pine is considered a keystone species as it provides a broad range of critical ecosystem services including regulation of spring and summer snowmelt vital to stream flow and irrigation practices (Farnes 1990), reduced soil erosion at certain sites (Weaver 2001), and its seeds are a major food source for many animals (Ellison et al. 2005, Lantz 2010). As is characteristic of stone pines, whitebark pine produces

indehiscent cones that contain numerous large wingless seeds that weigh approximately 175 milligrams and contain 52% fat and 21% protein by weight (Lanner and Gilbert 1994). These nutrient-rich pine nuts are evidence of coevolution with and dependence on animals for seed dispersal; in contrast, a majority of North American conifers produce dehiscent cones that contain wind-dispersed winged seeds (Tomback 1978, Hutchins and Lanner 1982). At maturity, cones are dark purplish brown, 5-8 cm long, and contain an average of 45 (Tomback 1982) to 75 (Weaver and Forcella 1986) seeds per cone, depending on the vigor of the yearly cone crop. Whitebark pine trees begins to produce cones around 20 to 30 years of age (Krugman and Jenkinson 1974).

Many animal species harvest whitebark pine seed including several species of birds, chipmunks, squirrels, and bears, but by far the most prodigious and efficient harvester of whitebark seeds is the Clark's nutcracker (*Nucifraga Columbiana* Wilson, Family Corvidae; Hutchins and Lanner 1982). The relationship between whitebark pine and the Clark's Nutcracker is likely a mutualistic coevolution, and alternative seed dispersers do not cache seed effectively for reproduction (Hutchins and Lanner 1982, Tomback 1982). The nutcracker's bill is long and sharp, designed specifically for opening the tough cones of whitebark and to reach the seeds inside (Tomback 1978). The birds have a unique throat pouch that is ideal for transporting many seeds at a time to a caching site (Bock et al. 1973). In one study, nutcrackers were observed to carry between 35-150 seeds in their pouches (Tomback 1978). In mid to late August whitebark trees begin to develop mature seed and nutcrackers begin harvesting and caching large amounts of seed, ultimately to be used as a food source when retrieved the following

spring and summer (Tomback 1978, Hutchins and Lanner 1982). On average, nutcrackers store 3-5 seeds per cache, but this range froms 1-15 per cache, planted at a depth of 1-3 cm (Hutchins and Lanner 1982, Tomback 1982). Nutcrackers have been observed to cache seed up to 32 km from the seed source, but more often they cache on steep south-facing slopes only 3 or 4 km from the source (Tomback 1978, Lorenz et al. 2011). Nutcrackers prefer to cache seeds in areas with visual cues that provide microsites such as burn sites; microsites are protective objects which vary from the bases of trees, rocks, roots, and fallen trees to open terrain or the edges of meadows (Hutchins and Lanner 1982, Tomback 1986, Tomback 2001). It is estimated that nutcrackers store 3 to 5 times the amount of seed needed to meet their energy requirements for the following season and that 45% of seed caches are not recovered (Tomback 1982). These unretrieved seed caches serve as the single dominant source of whitebark pine regeneration (Tomback 1982), and these caches effectively drive the genetic structure, range expansion, and community development of whitebark pine ecosystems (Tomback 2005).

While whitebark pine relies solely on the Clark's nutcracker for natural regeneration, the nutcracker is an opportunistic forager (Tomback 1998); recent research suggests that the significance of cached whitebark pine seeds as a food source for nutcrackers in the spring season may be overestimated. Schaming (2016) found that cached whitebark pine seed only accounted for approximately 9% of the nutcrackers' diet during the 2012 breeding season in study sites in the Greater Yellowstone Ecosystem. However, the importance of cached seed to the nutcracker's diet may depend on the region and on the availability of the previous season's cone crop (Schaming 2016).

Squirrels and bears are also major consumers of whitebark pine seed. Hutchins and Lanner (1982) observed that after the Clark's Nutcracker, red squirrels were the most commonly observed vertebrate to harvest in whitebark pine stands. Pine seeds, where available, are a major component of grizzly bear and black bear diets, along with ungulates and graminoids (Kendall 1983, Mattson et al. 1991, Fortin 2011); bears acquire pine seed primarily by raiding squirrel cone middens (Hutchins and Lanner 1982, Kendall 1983).

Whitebark Pine and Mycorrhizal Fungi

In addition to having close ties to several animal species, whitebark pine is an obligate associate of ectomycorrhizal (ECM) fungi, and the trees rely on this mutualism for growth and development (Read 1998, Smith and Read 2008). Ectomycorrhizal fungi form structures called mycorrhizae on the root tips of plants (mainly trees and shrubs) that aid in the uptake of nutrients such as nitrogen and in turn receive photosynthetically derived carbohydrates from the plant (Allen et al. 2003). Ectomycorrhizae are formed by the creation of a fungal layer called a mantle over the surface of the root tip and the development of a Hartig net; the latter is formed when fungal cells grow into the space surrounding root cells forming the plant-fungal interface for nutrient exchange (Brundrett et al. 1996, Smith and Read 2008). Fungal hyphae spread out from the mycorrhizal root tip into the soil creating a vast mycelial network that greatly surpasses the original surface area of the plant root available for nutrient and water uptake (Brundrett et al. 1996).

It is now estimated that over 85% of all plant species form arbuscular or ecto-mycorrhizal associations (Allen et al. 2003) and thousands of species of fungi belonging to Basidiomycota, Ascomycota, and Zygomycota are capable of forming these relationships (Molina et al. 1992, Allen 2003). Some ECM fungal species are considered generalists because they are able to form associations with several different plant species, while others are obligate associates of specific plant species (Smith and Read 2008). Some conifers such as Douglas fir associate with over 2,000 species of ectomycorrhizal fungi (Trappe 1962), but whitebark pine, a five needle stone pine, associates with fewer species of fungi (Mohatt et al. 2008). One study which sampled sporocarps and ectomycorrhizal root tips from whitebark pine forests across five mountain ranges in the Greater Yellowstone Ecosystem (GYE) revealed less than 50 species of EM fungi in association with whitebark pine; some of the more prominent groups of fungi found in association with whitebark pine were those in the cortinarioid, suilloid, russuloid, and thelepheroid clades (Mohatt et al. 2008, Cripps and Antibus 2011).

The suilloid species found with whitebark pine are known to be close associates of pines, five-needle pines, or stone pines in the family Pinaceae (Bruns et al. 2002a, Grubisha et al. 2002, Cripps and Antibus 2011). The suilloid clade is made up of ectomycorrhizal species in the genera *Suillus*, *Rhizopogon*, *Chroogomphus*, *Gomphidius*, and *Truncocolumella* (Bruns et al. 1989), and the first three genera are reported with whitebark pine (Mohatt et al. 2008). Epigeous *Suillus* and hypogeous *Rhizopogon* (false truffles) genera are both monophyletic, being highly derived from the Boletaceae family (Bruns et al. 1989, Grubisha et al. 2002), and species have a strong propensity to be host

specific (Grubisha 1998). This surprisingly close relationship has been explored through molecular work and the stark morphological differences (epigeous versus hypogeous) likely originate from an accelerated divergence due to environmental selective pressures (Bruns et al. 1989).

There are approximately 100 *Suillus* species worldwide and many are well known ectomycorrhizal associates of conifers, and in particular species in *Pinus*; approximately 20 species associate with 5-needle pines (Klofac 2013). One study showed that disjunct North American and Asian *Suillus* species form mycorrhizae with five-needle pine species originating from a monophyletic group, indicating that there are ecological and phylogenetic connections across broad biogeographic patterns (Wang et al. 1999, Wu et al. 2000). *Suillus sibiricus* Singer in particular is a global associate found with stone pines in the Swiss Alps, the Altai Mountains in central Asia, and the Rocky Mountains of North America (Moser 2004). In the northern Rocky Mountains *Suillus sibiricus* (Singer) Singer (= *S. americanus* (Peck) Snell), *Suillus subalpinus* M.M. Moser, and *Suillus discolor* (A.H. Sm, Thiers & O.K. Miller) N.H. Nguyen have been collected and identified with whitebark pine (Mohatt et al. 2008, Cripps and Antibus 2011). *Suillus* species occur in mature forests as shown by the collection of fruiting bodies (Mohatt 2008), and are significant colonizers of whitebark pine seedlings (Mohatt 2006, Trusty and Cripps 2011), indicating the importance of these fungi in all stages of the whitebark pine life cycle.

The hypogeous *Rhizopogon* species found with whitebark pine (*R. evadens* A.H. Smith and *R. milleri* A.H. Smith) are less specific and can also occur with two- and three-

needle pines (Grubisha et al. 2002, Mohatt et al. 2008). Not only are suilloid fungi an integral component of the whitebark pine life cycle, but these fungi are a significant food source for bears (Fortin 2011), deer (Ashkannejhad and Horton 2006), and small mammals such as squirrels and rodents (Maser and Trappe 1978, Maser and Maser 1988, Izzo et al. 2005), further tying these fungi to these unique ecosystems.

Fire

Wildfire is an integral part of northern Rocky Mountain forest ecosystems (Habeck and Mutch 1973) and is an important force in whitebark pine communities (Arno and Hoff 1989). This is especially true in seral communities where whitebark pine may be outcompeted and replaced by more shade tolerant and less fire resistant conifer species without periodic wildfire (Arno and Hoff 1989, Morgan and Bunting 1989). Wildfire plays somewhat less of a role in climax whitebark pine communities where competing tree species are less hardy and fire return intervals are much longer (Arno 2001). Keane et al. (2012) refers to a "high-mountain ecological triangle" in which whitebark pine, the Clark's nutcracker, and wildfire cooperate to perpetuate the diversity of these mountain ecosystems. The unique dispersal ecology of whitebark pine gives the species a competitive pioneering advantage post-fire as the Clark's nutcracker is able to disperse seeds longer distances than wind-dispersed conifers (Tomback 1982, Lorenz et al. 2008). The birds often prefer to cache seed on burn sites as these areas offer large open spaces with visual cues and a variety of microsites such as downed trees and logs (McCaughey 1990, Tomback 2001a). Tomback et al. (1993) found that at burn sites, 81% of whitebark pine seedlings grow within 15 cm of a microsite. These cached seeds have a

better chance of germination and survival once the area has been cleared of competition by wildfire (Keane et al. 2012).

Moody (2006) found in a study of burned and non-burned paired sites that most often, natural regeneration of whitebark pine seedlings was greater in burned sites (though not always) and that seedling regeneration in burned sites was also highly dependent on seed source distance, size, and density. Larson and Kipfmueller (2010) found natural regeneration to be comparable in burned and un-burned sites, but sites were not paired for similarity. Klutsch et al. (2015) found that while whitebark pine seedling regeneration density was lower in burned sites when compared to adjacent unburned sites, seedlings in the burned sites were much younger on average and had a growth rate 1.6 times higher than those in the unburned sites; the researchers hypothesized that the lower densities in the burn sites could be partially caused by delayed germination. Another study which surveyed 15 burn sites within the elevational range of whitebark pine in the northern Rocky Mountains found that regeneration at the majority of sites was dominated by whitebark pine and that germination of seed was often delayed for several years, with 73% of seedlings germinating 5-10 years post-fire (Leirfallom 2014). Tomback (2001b) also found that the majority of whitebark pine regeneration on burn sites was delayed by at least 2 years for up to 7 years after the 1988 Yellowstone fires. The majority of regeneration was observed 3 and 5 years after the fires and this was not predictable from the previous seasons' cone crops. This delay of germination effectively creates a soil seed bank and germination of whitebark pine seedlings will continue over years in a burn site (Tomback 2001b). This delay of germination in addition to nutcracker

dispersal gives whitebark pine an advantage over other wind-dispersed conifers in initial post fire regeneration as well as a continuity of germination.

Threats

Within the past few decades, whitebark pine has been declining throughout the majority of its range, with an overall decline of up to 90% in some areas (Kendall and Keane 2001, Keane et al. 2012). This is due to a number of factors including mountain pine beetle (*Dendroctonus ponderosae* Hopkins) outbreaks, white pine blister rust (*Cronartium ribicola* Fisch.) invasion, climate change, and fire suppression. Whitebark pine is considered a keystone species and as such its loss could result in a cascade of damaging effects throughout these unique ecosystems (Ellison et al. 2005).

The principal threat to the existence of whitebark pine is the invasion and spread of white pine blister rust (WPBR), an invasive fungal pathogen introduced to forest nurseries in North America from Eurasia around 1900 (McDonald et al. 2001). WPBR has a complicated life cycle and it alternates mainly between white pine (*Strobus*) hosts and woody shrub species of *Ribes* (Grossulariaceae), cycling through 5 stages of spores to complete one generation (McDonald et al. 2001). Less common telial hosts include *Pedicularis* and *Castilleja* in the Orobanchaceae (McDonald et al. 2006). Whitebark pine is highly susceptible to WPBR and some areas have seen up to a 100% infection of forests (Keane et al. 2012). The fungus enters the needles through the stomata and girdles and kills limbs, often first attacking the topmost cone-bearing branches resulting in a loss of viable seed (Keane et al. 1994, McKinney and Tomback 2007). One study that examined 17 whitebark pine plots established in 1971 in western Montana found a

dramatic increase in mortality rates and blister rust infection 20 years later; overall, plots had an average infection rate of 89% and the average mortality rate was 42% (Keane and Arno 1993). Another more recent study that examined 115 plots in the Canadian Rocky Mountains saw an overall increase in infection of live trees from 42% in 2003-2004 to 52% in 2009 (Smith et al. 2012). The range of WPBR now nearly encompasses all of the range of whitebark pine including B.C and Alberta in Canada and all of the western United States except for Utah (Schwandt et al. 2010), and the fungus has been found in even the coldest and driest climax whitebark pine communities (Kendall and Keane 2001, Resler and Tomback 2008). There does appear to be a geographic trend with infection rates generally increasing from the north to the south possibly indicating an increase in resistance with latitude (Mahalovich et al. 2006, Smith et al. 2012).

The mountain pine beetle (MPB) is native to North America but recent increases in population outbreaks driven by the warming climate (Bentz and Schen-Langenheim 2007) are causing unprecedented mortality of high elevation pines, with almost half a million acres of whitebark pine beetle kill in 2007 (Gibson et al. 2008). These beetles preferentially attack mature, often cone-bearing trees (Kendall and Keane 2001) so this widespread mortality has the potential to greatly reduce the seed available for regeneration. Trees already weakened by WPBR are more likely to be attacked by mountain pine beetles (Six and Adams 2007, Gibson et al. 2008). One study conducted in three whitebark pine stands in the GYE showed definite interactions between WPBR and MPB, with rust severity scores of trees selected by MPB being almost twice as high as those trees that were not selected (Bockino and Tinker 2012). Beetle-killed trees may

serve as fuel for wildfire, an important recycling force in pine forests (Schmidt 1988, Logan and Powell 2001), but overall effects of beetle-killed stands on fire regimes are dependent upon stand condition. Beetle attacks at differing stages have the potential to alter fire severity under moderate burning conditions (Harvey et al. 2014), but likely do not increase the actual likelihood of wildfire occurrence (Meigs et al. 2015).

For the majority of the 20th century, a "culture of suppression" ruled wildfire policy, and the ecological importance of wildfire was left largely unrealized until late in the century. Still today land managers pursue a delicate balance between wildfire monitoring and suppression (Aplet 2006). Historically, fire intervals in whitebark pine forests ranged from 30 to 300 years; fires often burned in mixed-severity patchy patterns and sometimes at a stand replacing level, which benefitted whitebark persistence and regeneration (Arno 2001). However, recent fire suppression has resulted in an increase in these return intervals and an overall reduction in area burned. One study revealed that presettlement (pre-1935) fires in the Selway-Bitterroot Wilderness of Idaho and Montana burned approximately 1.5 times the area of more recent fires (1979-1990; Brown et al. 1994). This suppression of natural wildfire has caused a decrease in seral whitebark pine populations that rely on periodic wildfire to maintain a presence in mid-elevation mixed forest communities (Arno 2001). In the Bitterroot National Forest, whitebark historically made up approximately 39% of the basal area of seral subalpine forests, but decreased to only 11% in 1995 (Hartwell 1997).

Finally, climate change is a direct threat to whitebark pine in addition to exacerbating the effects of the previously discussed threats. Several studies utilizing

bioclimatic envelope modeling have projected a dramatic decrease in suitable climatic habitat for whitebark pine in the next several decades (Warwell et al. 2006, Chang et al. 2014). Warming climate also results in increases in MPB populations and longer and more intense episodes of attack (Logan and Powell 2001, Logan et al. 2010). The spread of WPBR itself may also be driven by a warming climate due to the climatic conditions pivotal to the blister rust life cycle (Koteen 2002). All of these threats interact to make whitebark pine particularly vulnerable to climate change, adding a level of complexity to restoration efforts and highlighting the need for adaptive management strategies (Hansen et al. 2016).

The rapid decline of whitebark pine populations likely holds dire implications for the ecosystems surrounding this keystone species. Over the past few decades in the Greater Yellowstone Ecosystem (GYE) the annual rate of population growth for grizzly bears has slowed and some studies have correlated the decline of whitebark pine with grizzly bear survival (Haroldson et al. 2006) and movement (Blanchard and Knight 1991). However, recent studies suggest that the decline in population growth and decrease in home-range size may be due to population density rather than whitebark pine mortality (Bjornlie et al. 2014, van Manen 2016). Regardless, while dependent upon seasonal and annual variation, whitebark pine seeds are a significant portion of the grizzly bear diet where the species' ranges overlap (Kendall 1983, Mattson et al. 1991), and the loss of this food source would likely have negative consequences for the grizzly bear. The decline of whitebark pine may also have consequences for the closely associated Clark's nutcracker. A recent study conducted in the GYE found that in 2 out of

5 years there was a population-wide failure to breed in Clark's nutcrackers and that these failed spring breeding seasons followed fall seasons with very low whitebark cone crops; researchers hypothesized that the failure to breed was likely due to either low numbers of cached seed or low stored body energy levels (Schaming 2015).

Restoration

The compilation of threats both natural and anthropogenic and the resulting loss of whitebark pine has led scientists and land managers to actively pursue a strategy for restoration of this keystone species. A range-wide strategy for restoration has been developed by leading managers in the field and focuses on promoting rust resistance, conserving genetic diversity, saving seed sources, and employing restoration treatments (Keane et al. 2012). These strategies are applied across the range of whitebark pine and rely on the collaboration of land managers, scientists, and academics.

Seed Source

The most promising strategy for restoration of whitebark pine is the out-planting of blister rust resistant seedlings (Keane et al. 2012). Due to the continuous loss of mature cone-bearing whitebark pine, it is necessary to collect seed for blister rust resistance screening, genetic conservation, and out-planting. Cones are collected preferentially from healthy cone producing whitebark trees in forests with high mortality from white pine blister rust (Burr et al. 2001). Burr et al. (2001) recommend that cone collection sites exhibit at least a 50 percent mortality rate due to WPBR and that individual trees selected for seed collection, or plus trees, ideally have no cankers, or if

no trees that meet the criteria can be found, five or less cankers. Once plus trees are identified, second-year cones can be protected from predators by placing cages around the cone-bearing branches in June and leaving the cages until collection in the fall when seeds have fully matured. Cones are collected in fall by tree climbers, stored in burlap sacks, and are quickly transported to extractor facilities; it is essential to keep cones dry and cool during transport (Burr et al. 2001).

Seed collection is divided into seven seed zones and the out-planting location of resulting seedlings should be consistent with the seed zone of origin in order to avoid maladaptation (Mahalovich and Dickerson 2004). Recent research utilizing spatial overlays of the range of whitebark pine, soil parent material type, and isotopic composition of sampled pine nuts revealed strong correlations between these factors (Mahalovich et al. 2016). This information has the potential to play a significant role in the selection of appropriate seed sources for future plantings and in ensuring seedling survival and vigor (Mahalovich et al. 2016).

Seedlings

Whitebark pine seedlings destined for out-planting in the Rocky Mountain area of the United States are primarily grown at the Coeur d'Alene Forest Service Nursery in Idaho according to a specific protocol for germination and growing (Burr et al. 2001). Cones collected in the field are delivered to the nursery and are dried slowly at room temperature; then seeds are separated from cone scales by hand. Seed is inspected at random for quality and potential for germination utilizing a non-destructive x-ray and is then either stored for future use or enters the germination process for seedling

propagation. Now, with proper timing of cone collection and sufficient drying, whitebark pine seed can be stored for several years at or just below freezing prior to germination (Robertson et al. 2013). A specific stratification protocol has been developed for whitebark pine seeds in order to achieve high levels of successful germination in the nursery, and the current practice is as follows: 48 hour running water soak to reduce risk of fungal pathogens, 28 day warm stratification with 12 hour photoperiod, 60 day cold dark stratification with weekly soaks (Burr et al. 2001). Following the current practice, two non-scarified seeds are planted directly in Ray Leach Super Cells in order to increase chances of producing at least one seedling per container (Stuewe and Sons, Inc., Corvallis, Oregon) (Eggleston 2010). Seedlings at the Coeur d'Alene Forest Service Nursery are currently grown under standard greenhouse conditions in a media of ground peat moss and composted Douglas-fir bark (Robertson 2015).

Blister Rust Resistance Screening

In order to test plus tree progeny for inherited blister rust resistance, a portion of seedlings grown in the nursery are submitted to an artificial rust inoculation process (Hoff et al. 2001, Mahalovich et al. 2006). For the inoculation, *Ribes* leaves infected with WPBR are placed on screens above seedlings inside inoculation chambers under controlled humidity and temperature. Microscope slides are also placed beneath the *Ribes* leaves for the purpose of counting and ensuring that targeted spore drop numbers are reached. After inoculation, seedlings are monitored closely for signs of resistance including needle lesion frequency, needle drop, early stem symptoms, bark reactions, and canker tolerance (Hoff et al. 2001, Mahalovich et al. 2006). Researchers hope to find a

combination of resistance types to breed for a ‘durable’ resistance in whitebark pine, with the aim to avoid combative evolution of blister rust (Sneizko et al. 2011).

Promising levels of resistance have been observed in many seed sources within all seed zones and genetic stock is continually being selected and retested for resistance through multiple inoculations. Progeny from plus trees ranking high in rust resistance are selected for the purpose of out-planting in the field. One observed trade-off is that seedlings with a higher WPBR resistance appear to be somewhat less cold-hardy, possibly due to necessary allocation of resources within the trees (Mahalovich et al. 2006). Resistant progeny is also being used to establish seed orchards for the continual production of resistant seed as part of the Forest Service breeding program in the interest of maintaining genetic diversity (Mahalovich et al. 2006).

Outplanting

Thousands of whitebark pine seedlings are planted every year on public lands in the western U.S. and Canada (Izlar 2007). The collection of seed, screening for blister rust resistance, and production of seedlings for these plantings is an expensive and involved process, and the cost is now approximately $2.00 per seedling (Waring and Goodrich 2012). Therefore, it is important to explore how the practice of out-planting can be improved to achieve the highest possible survival rates in order to optimize the use of these valuable seedling resources. General guidelines have been developed for the out-planting of whitebark pine seedlings (McCaughey et al. 2009, Scott et al. 2011) but methods are continually being tested and long term monitoring will help determine the best practices. Recommendations for planting sites include areas with reduced overstory

and understory competition, on ridgetops and slopes where whitebark has grown in the past, and where there is adequate soil moisture (McCaughey et al. 2009). Seedlings should be healthy with vigorous root systems and should be planted with a microsite such as a stump, log, broken snag, rock, or downed tree for protection from heavy snow, winds, and light intensity. Small logs or tall snags that could fall on the seedling should be avoided and when possible microsites should not be placed downhill of seedlings on a steep slope as soil runoff can bury small trees (McCaughey et al. 2009).

Surrounding vegetation should be taken into consideration when planting, as some studies have correlated seedling survival and growth rates with understory vegetation. For example, Perkins (2015) found that seedling growth and survival rates decrease when planted next to beargrass (*Xerophyllum tenax* (Pursh) Nutt.) or Geyer's sedge (*Carex geyeri* Boott.), likely due to the dense root masses that these plants produce (Landhäusser et al. 1996); survival rates increased when they were planted next to grouse whortleberry (*Vaccinium scoparium* Leiburg ex Coville).

Recent burns are often recommended as planting sites for whitebark pine seedlings as they provide large areas cleared of competition with a variety of microsites (Keane et al. 2012). In natural regeneration of whitebark pine, the Clark's nutcracker often preferentially caches seed on burn sites, but natural regeneration may depend directly on the health of surrounding forests and seed source (McKinney and Tomback 2007). One study found that natural regeneration in burn sites was best predicted by the health of mature trees in adjacent stands, and that regeneration on a burn was low if <50% of the mature trees in the adjacent stand were healthy (Leirfallom 2014). Other

studies have shown that a decrease in whitebark pine stand health and cone production can result in fewer visitations from nutcrackers (McKinney and Tomback 2007, Barringer et al. 2012). It is now recommended that sites with >20% rust-caused mortality and >50% rust infection or sites with high mortality from beetle kill be replanted with blister rust resistant seedlings since natural regeneration may not materialize (Keane and Parsons 2010).

Many studies have monitored natural regeneration in burn sites as discussed earlier (Tomback et al. 2001b, Moody 2006, Larson and Kipfmueller 2010, Klutsch et al. 2015), but comparatively few have monitored the success of out-planting of nursery seedlings on burn sites as current restoration practices recommend. Izlar (2007) conducted the largest monitoring project to-date for out-planted whitebark pine seedlings, visiting 48 sites and approximately 114,000 seedlings, and found that survival rapidly decreased from 74% in the 1st year to 38% in years 3-15; however, long term survival was significantly greater when seedlings were planted in mixed (52%) or severely burned (41%) sites when compared to those planted in moderately burned (29%) or unburned (21%) sites. Perkins (2015) found that seedling survival was nearly 150% greater, biomass more than doubled, and foliar nitrogen content was 34% greater for seedlings in burned sites compared to unburned sites. However, this study utilized the direct planting of seeds instead of seedlings and germination rates were very low overall, with only 11% of seeds germinating by the second season (Perkins 2015). Whether this apparent benefit of planting on a burn site results from the lack of competition or the ‘pulse’ of nutrients purported to be released by fire (Certini 2005) is not known.

Mycorrhizal Fungi

In pursuing restoration of a species, the entire natural system surrounding the species needs to be taken into account. Mycorrhizal fungi are an integral part of the life cycle of whitebark pine and should be considered as a tool for restoration in out-plantings (Keane et al. 2012). This is especially true for host-specific *Suillus* fungi such as *S. sibiricus*, *S. discolor*, and *S. subalpinus* that associate primarily with five-needle pines including whitebark pine (Mohatt et al. 2008, Cripps and Antibus 2011). Evidence points to a long co-evolutionary history between *Suillus* fungi and *Pinus* species (Wu et al. 2000), and because species in these genera are so closely tied, the decline of whitebark pine could also result in a decline of the associated fungi that these forests support (Mohatt et al. 2008, Karst et al. 2014). Preservation of the fungi themselves could be critical to the maintenance of whitebark pine forests.

Certain suilloid fungi are associated with other stone pines around the world and have been successfully used as a tool in their restoration (Moser 2004, Weisleitner 2008). In Austria, *Suillus* species including *S. plorans* (Rolland) Kuntze, *S. sibiricus* Singer, and *S. placidus* (Bonord.) Singer have been successfully utilized in restoring populations of high elevation European Stone pines (*Pinus cembra* L.) through inoculation and out-planting of nursery grown seedlings. Seedlings inoculated with these *Suillus* species exhibited a 20-80% increase in survival and approximately 30 years after planting, mycorrhizae collected from the roots were identified as those from the original inoculants (Moser 1956, Schmid 2006). A more recent study evaluating the ectomycorrhizal community of these out-planted pines revealed that 82% of sampled root tips were

colonized by the original inoculants indicating that the inoculation has been sustainable over decades (Rainer et al. 2015). The Federal Forest Nursery in Austria has now been inoculating seedlings for over 50 years and maintains colonization in the nursery by growing seedlings in biodegradable pots that allow roots of neighboring seedlings to interact resulting in a continual spread of the fungi throughout the nursery (Weisleitner 2008). Due to differences in climate and nursery practices, new types of inoculation methods for whitebark pine are being developed in the United States (Cripps and Grimme 2011, Lonergan and Cripps 2013).

Greenhouse Studies Utilizing Suilloid Fungi

Inoculation trials at Montana State University that tested 25 strains of ectomycorrhizal fungi native to whitebark pine forests identified *Suillus* species as vigorous colonizers of seedlings in the greenhouse. The most effective type of inoculum is fresh spore slurry created by grinding the hymenium (spore producing tissue) of fresh fungal sporocarps with water and adding the slurry directly to seedling containers (Cripps and Grimme 2011). Fertilization regimes need to be taken into consideration in inoculation of seedlings in a greenhouse as routine fertilizer application has been shown to suppress mycorrhizal colonization of whitebark pine seedlings (Cripps and Grimme 2011). Lonergan and Cripps (2013) found that application of a very low nitrogen fertilizer, Phosgard 4N:25P_2O_5:15K_2O liquid NPK fertilizer (JH Biotech Inc, Ventura, California) at a 13 ppm solution once every other week, still allows for colonization of whitebark pine seedlings in a greenhouse setting. This experiment also tested inoculation method, comparing a “drip” method in which spore slurry is dripped over the entire root

system to an injection method in which a syringe is used to inject spore slurry directly into the seedling container; while no significant difference in mycorrhizal colonization was observed between the two methods, the injection method proved to be much more efficient and involved less exposure of seedling root systems (Lonergan and Cripps 2013).

A variety of studies have shown that pine seedlings inoculated with suilloid fungi may benefit in a greenhouse setting (Table 1.1). Blue pine (*P. wallichiana)*, a five-needle pine, inoculated with *S. sibiricus* showed an increase in growth and total biomass (Verma et al. 2014). Similarly, *P. sylvestris* seedlings inoculated with *S. luteus* also showed an increase in biomass in the greenhouse (Hobbie and Colpaert 2003). Ectomycorrhizal fungi including suilloid fungi also have the potential to benefit seedlings through the uptake of organic forms of nitrogen not typically available to plants (Hobbie and Högberg 2012). European stone pines (*P. cembra*) inoculated with *S. placidus* (Heumader 1992) and *P. halepensis* seedlings inoculated with *S. collinitus* (Rincon et al. 2007) showed higher foliar nitrogen contents when compared to un-inoculated seedlings. Nutrient status and biomass are important indicators of morphological and physiological fitness, and are indicative of the out-planting performance of seedlings produced in nurseries (Haase 2008, Grossnickle 2012).

While several studies have investigated the effects of inoculation with suilloid fungi on pine seedlings in a greenhouse setting, few have considered the combined effects when planting in soil from a burn site. Sousa et al. (2011) planted *P. pinaster* seedlings inoculated with *S. bovinus* in heat-treated soil in a greenhouse and saw a

resulting increase in growth and shoot fresh weight when compared to control seedlings. As current practices for the restoration of whitebark pine include out-planting on burn sites, more research is needed in a greenhouse setting to provide insight into field performance.

Nutrient Analysis and Isotopic Patterns

Mycorrhizal fungi directly influence isotopic patterns in plants and these patterns may provide useful insight into the exchange of nutrients. *Suillus* species are primarily involved in the uptake of nitrogen (Keller 1996) and this can be explored by measuring ^{15}N isotopic signatures of the fungal fruiting body as well as the needles of the pine (Hobbie and Colpaert 2003). Natural abundances of isotopes in a substance, represented as δ, are calculated as the ratio of the heavier to lighter isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$) in the sample relative to an internationally designated standard (Dawson et al. 2002). In the assimilation of nitrogen, fungi preferentially retain ^{15}N, and pass fractionated nitrogen to the plant. Therefore, shifts in foliar $\delta^{15}N$ values tend to reflect the mycorrhizal status of a plant's root system and seedlings colonized by mycorrhizal fungi in a greenhouse setting will exhibit lower foliar $\delta^{15}N$ values compared to uncolonized seedlings (Kohzu et al. 2000, Hobbie and Colpaert 2003). This has been shown in several studies utilizing suilloid fungi. Foliar $\delta^{15}N$ was significantly lower and strongly inversely correlated with seedling biomass in *Pinus sylvestris* seedlings inoculated with *S. luteus* in comparison to un-inoculated seedlings (Hobbie and Colpaert 2003). Similarly, *P. densiflora* seedlings inoculated with *S. granulatus* also had significantly lower foliar $\delta^{15}N$ values and higher foliar N content in comparison to un-inoculated seedlings (Kohzu et al. 2000). Together,

these studies show that inoculation with *Suillus* species can result in greater seedling vigor and a simultaneous decrease in foliar $\delta^{15}N$, indicating that plant-acquired nitrogen is obtained from the mycorrhizal fungi. Therefore, the application of mycorrhizal fungi can potentially reduce fertilizer costs as well as protect seedlings from root pathogens in a greenhouse setting (Whipps 2004).

These studies all defined inoculation of seedlings as the explanatory variable in order to analyze seedling responses. However, an important factor often overlooked is that inoculation does not always result in colonization and to truly explore the effects of mycorrhizal colonization, it may be necessary to assess which seedlings actually became colonized after inoculation. The direct effects of colonization by *Suillus* spp. on pine seedlings appear to result in overall increased vigor which could provide seedlings with a "jump-start" before being out-planted (Davey 1990).

Table 1.1: Response of pine seedlings to mycorrhizal inoculation with *Suillus* species summarized from various greenhouse studies.

Pinus spp.	*Suillus* spp.	Seedling Response to Inoculation	Source
P. wallichiana	*S. sibiricus*	+ growth, + biomass	Verma et al. 2014
P. cembra	*S. placidus*	+ foliar N content	Heumader 1992
P. haplensis	*S. collinitus*	+ foliar N content	Rincon et al. 2007
P. sylvestris	*S. luteus*	- foliar $\delta^{15}N$, + biomass	Hobbie & Colpaert 2003
P. densiflora	*S. granulatus*	- foliar $\delta^{15}N$, + foliar N content	Kohzu et al. 2000

Carbon isotope signatures can also be used to make inferences about physiological processes in plants. Measures of $\delta^{13}C$ are most often used to estimate plant water-use efficiency (WUE; Farquhar et al. 1989, Adams and Grierson 2001). $\delta^{13}C$ values are directly related to the concentration of CO_2 in leaves which in turn is indicative

of WUE, thus it is often extrapolated that higher foliar $\delta^{13}C$ indicates greater WUE (Gower and Richards 1990). However, researchers acknowledge that WUE is more complicated than this and can be affected by variation in plants both genotypical and phenotypical (Farquhar et al. 1989). Few studies have investigated correlations between $\delta^{13}C$ and ectomycorrhizal colonization. Hobbie and Colpaert (2004) hypothesized that $\delta^{13}C$ of *Pinus sylvestris* seedlings would increase with ectomycorrhizal colonization by *Suillus luteus* (L. Fr.) Roussel due to increased photosynthetic rates triggered by the carbon sink created by mycorrhizal colonization of roots (Dosskey et al 1990). Results from this study revealed that seedlings colonized by *S. luteus* at low N availability were enriched in foliar $\delta^{13}C$ by approximately 1‰ when compared to non-mycorrhizal seedlings or seedlings colonized by *Thelephora terrestris* (Ehrh.) Fr. (Hobbie and Colpaert 2004); this was attributed to enhancement of photosynthetic rates due to colonization as shown by a previous study (Colpaert et al. 1996).

Inoculation and Outplanting on Burns

Inoculation of whitebark pine seedlings with ectomycorrhizal fungi should be considered when out-planting, but may not always be necessary (Cripps and Grimme 2011). If planting is in an area previously occupied by whitebark pine, seedlings could potentially become naturally colonized by the native fungi present in soil spore banks. However, even in areas where native inoculum may be present, inoculation in the nursery could benefit seedlings in the critical establishment period immediately post planting (Ortega et al. 2004, Quoreshi et al. 2009). If planting in an area likely lacking a viable spore bank such as a severe burn, a ghost forest where no living trees remain, or an area

where whitebark pine was not previously present, inoculation with appropriate mycorrhizal fungi may be necessary. The area adjacent to plantings needs to be taken into consideration as well, and this includes the health of the surrounding forest and distance to a potential fungal inoculum source (Cripps and Grimme 2011). Planting in an area with healthy adult whitebark pine nearby encourages the dispersal of native fungi into the planted area through the feces of animals feeding on sporocarps (Ashkannejhad and Horton 2006, Trusty and Cripps 2011).

Recent recommendations for the restoration of whitebark pine suggest combining inoculation of seedlings with native ectomycorrhizal fungi and planting on burn sites (Keane et al. 2012). Moderate- to severely burned sites can provide large areas cleared of competition as well as a plethora of microsites to plant seedlings in mimicry of the Clark's nutcracker. However, burn sites may lack viable fungal spore banks and may require inoculation of seedlings for re-establishment of a mycorrhizal fungal community especially on severe burns (Wiensczyk et al. 2002). The resistance of fungal propagules to fire is species dependent and depends on the severity of the fire, but the general trend is toward a decrease in diversity of ectomycorrhizal fungi directly after wildfire (Smith et al. 2005, Cairney and Bastias 2007). The persistence of suilloid fungi in soil after fire has been examined by a number of studies. Bruns et al. (2002b) found that *Suillus pungens* Thiers & A.H. Sm. fungal genets present in post-fire soil did not match those of pre-fire individuals, which indicates a lack of surviving spores or mycelium; instead recolonization of the burn site was likely through wind-dispersed spores. Other studies have shown that some species of *Rhizopogon* tend to be fire or heat resistant while *Suillus*

species tend to decrease with fire or heat (Baar et al. 1999, Izzo et al. 2006, Peay et al. 2009, Glassman et al. 2015). Trusty and Cripps (2011) found a 40-60% reduction in ECM fungal diversity and a 10-13% reduction of suilloid fungi on whitebark pine seedlings roots five years after a severe fire in comparison to those in the immediately adjacent unburned forest; recolonization of the burned portion of this site by ECM was attributed to the nearby source of inoculum (mature whitebark pine trees) in the unburned portion of the study site as well as to the presence of animal vectors. Recolonization of a burned forest depends directly on the presence of pre-fire fungi as well as on the surrounding sources of inoculum for wind and animal dispersal. With the current state of widespread mortality of whitebark pine, it is more and more likely that areas designated to be planted may lack appropriate and available natural inoculum sources and artificial inoculation by native ectomycorrhizal fungi of out-planted seedlings should be considered (Keane et al. 2012). The use of commercial inoculum not specifically developed for whitebark pine risks the introduction of alien fungi into sensitive systems (Schwartz et al. 2006, Keane et al. 2012).

Ectomycorrhizal species are now widely used in the restoration of conifer species (Khasa et al. 2009). Several examples exist in which suilloid fungi, mainly *Rhizopogon* species, have been utilized in this sense (Castellano 1996, Amaranthus 2002, Steinfeld et al. 2003), but few of these include planting on burn sites. One study was found in which Douglas-fir seedlings showed increased growth when they were inoculated with *Rhizopogon parksii* and planted on logging debris burn piles (Teste et al. 2004). Even fewer studies have combined the inoculation of seedlings with *Suillus* species and out-

planting on burns. In Portugal, *P. pinaster* seedlings inoculated with ECM species including *S. bovinus* and *S. granulatus* resulted in increased growth (height and root collar diameter) five years after planting on a recent burn site (Franco et al. 2014).

Initial studies monitoring the effects of suilloid inoculum on the survival of whitebark pine seedlings planted on burn sites show promising results. One study conducted near Summit Lake in Waterton Lakes National Park, Alberta, Canada investigated the effects of a variety of treatment combinations including torching, planting in beargrass, planting in mircrosites, and with mycorrhizal inoculation (*S. sibiricus*) on the out-planting of almost 1,000 whitebark seedlings (Lonergan et al. 2014). Treatment combinations of all factors produced differing results, but overall seedling survival was highest when they were inoculated and planted on a torched area with a microsite. Three years after planting, approximately 11% of the increase in seedling survival was directly attributable to ECM inoculation, and this percentage increased when combined with planting on burn sites with microsites (Cripps et al. 2014).

Since 2002, Glacier National Park has planted over 20,000 whitebark pine seedlings across 14 sites, and over 4,000 of these seedlings have been monitored for at least one year (Asebrook and Hintz 2015). Two of these sites have included treatments of planting on burns and inoculation with native ectomycorrhizal *Suillus* species. The first of these sites, Divide Creek, was planted in 2010 and monitored over the next 5 years. After 4 years, there were no significant differences in height or survival in response to planting in the burn and with inoculation, but overall survival rates of seedlings were much higher (94%) than the average (62%) for plantings in the park. In the fifth year of

monitoring, inoculated seedlings had a slightly greater growth rate, though differences were still not statistically significant. The second of these sites, Divide Mountain, was planted in 2012 and monitored the following 3 years. Planting in the burned portion of the site and inoculation of seedlings resulted in an overall survival of 59%. The highest survival (90%) was observed for seedlings planted in the burn and inoculated with mycorrhizal fungi and lowest survival (30%) was observed for seedlings not planted in the burn which were not inoculated. Inoculation also improved survival by 20% when seedlings were not planted in the burn. The greatest change in height was also observed for seedlings planted in the burn that were inoculated with ECM fungi (+1.16 cm) (Asebrook and Hintz 2015).

Similar to the greenhouse studies mentioned earlier, one factor that all of these studies do not take into account is that inoculation does not always result in root colonization of a seedling and it may be that the genetics of individual seedlings play a role (Cripps and Grimme 2011). The lack of mycorrhizal assessment before out-planting is mostly attributable to a lack of time, resources, and expertise. However, in order to truly determine the effects of ectomycorrhizal colonization of seedlings, it is best to record actual colonization prior to out-planting.

GPS and GIS Technology for the Monitoring of Whitebark Pine

Currently, GPS and GIS technology are not widely used for the restoration and monitoring of whitebark pine. Researchers typically only use a GPS to mark the centers of plots or the beginning and ends of transects (Izlar 2007, Lonergan et al. 2014, Asebrook and Hintz 2015). Though time consuming, the spatial doumentation of

individual seedlings across a planting site establishes permanent long-term data sets that can be used in unique and powerful analyses. Spatial analyses provide a way to visualize data that can reveal patterns in health and survival that may otherwise not be noticeable in a list-form data set. Several studies have used spatial tagging to monitor the outplanting and natural regeneration of seedlings in various habitat types (Freeland et al. 2010, Wahungu et al. 2011, Miletti et al. 2005, Fei et al. 2010), but this has not been done for whitebark pine. Spatial documentation of plantings not only allows for unique analyses for the site documented, but is also a powerful predictive tool. If patterns in seedling health and survival correlate to planting variables derived from spatial datasets that are widely available for download and use, these data sets can be used to predict seedling survival at future planting sites. Land managers could use these methods to aid in the selection of appropriate planting sites with the best chance for seedlings success.

Restoration of Whitebark Pine in the Beaverhead-Deerlodge National Forest

The GYE represents an important portion of the range of whitebark pine, which represents 10% of the land cover. Additionally, all 22 mountain ranges are home to contiguous climax WBP communities (GYCC 2011, Macfarlane et al. 2013). Recently, the GYE has experienced an increase in whitebark pine mortality and aerial evaluation data has indicated that approximately 46% of whitebark pine stands in the GYE have experienced severe mortality from the mountain pine beetle alone (Macfarlane et al. 2013). One study found that 69% of whitebark pine trees tagged in 2002 for cone production monitoring in the GYE were dead by 2009 (Haroldson et al. 2011). In 2011, the Greater Yellowstone Coordinating Committee (GYCC) Whitebark Pine

Subcommittee released their "Whitebark Pine Strategy for the Greater Yellowstone Area" (GYCC 2011), a plan of action for the restoration of whitebark pine which stresses: collaboration of federal agencies, protection of remaining cone-producing whitebark pine, maintenance and restoration of WBP ecosystem functioning, genetic preservation and restoration of the species, and fire planning. The Beaverhead-Deerlodge National Forest (BDNF) spans over 900,000 acres of the GYE and whitebark is estimated to represent 15.6% or over 150,000 acres of the land coverage. Based on stand-level condition scores estimated from canopy damage and current mountain pine beetle action, a large portion of the whitebark pine forests in the BDNF hold a high restoration priority (GYCC 2011).

The Beaverhead-Deerlodge National Forest Plan provides guidelines for natural resource management and includes an objective to "promote regeneration of whitebark pine on approximately 45,000 acres, largely through the use of fire" (Olson 2015). The Eureka Basin Burn of 2013 was started by lightning strike on August 12th and burned primarily at high elevation mixed-conifer stands dominated by whitebark pine in the Gravelly Mountain range of southwestern Montana. In total, the fire burned a perimeter of approximately 6,468 acres and an estimated 44% of the burn was classified as moderate-high severity. The Eureka Burn provided a large area which was previously dominated by whitebark pine ideal for planting and restoration objectives. Over the next several years, land-managers of the BDNF plan to replant a large portion (up to ca. 1,400 acres) of the Eureka Burn area with approximately 40,000 blister rust resistant whitebark pine seedlings per year (Olson 2015). This large scale planting proposal for the

reforestation of whitebark pine in the Gravelly Mountains provides the opportunity to establish long term monitoring plots for the application and assessment of current planting strategies across a large heterogeneous natural burn.

Research Objectives

Currently, land managers recommend the out-planting of blister rust resistant seedlings on sites where the habitat is suitable for whitebark pine. This oftentimes includes the planting of seedlings on large-scale moderate to severe burns; these are sites where inoculation with native mycorrhizal fungi may be necessary to promote seedling establishment and to restore the ectomycorrhizal soil community. There is a widespread need for long term monitoring and collaboration on planting practices to determine the success of these plantings in regard to biotic (mycorrhizal colonization) and abiotic (site, microsite, etc.) factors.

This project aims to examine the effects of mycorrhizal colonization on whitebark pine seedlings planted in soil from a burn site with the overall goals of increasing seedling vigor and survival. A greenhouse study will explore the effects of colonization of whitebark pine seedlings by *Suillus* spp. in a greenhouse bioassay utilizing soil collected from the Eureka Burn site; seedling responses to experimental treatments that will be analyzed include: mycorrhizal assessment of root growth into soil mixtures, a DNA analysis of mycorrhizal species present, total seedling biomass, foliar nitrogen and carbon content, and foliar $\delta^{15}N$ and $\delta^{13}C$. A field study will monitor seedlings out-planted on the Eureka burn site for the effects of mycorrhizal colonization on seedling

health and survival, and will include a spatial analysis of abiotic factors (including microsite and Topographic Wetness Index) that might affect seedling survival. Integration of health monitoring (by colonization status) with a spatial analysis of abiotic factors utilizing ArcMap software has the potential to predict seedling survival for various regeneration strategies for land managers in the restoration of whitebark pine. The short-term controlled greenhouse study and the long-term applied field study will serve to provide unique insight into the effects of mycorrhizal colonization of seedlings planted on a burn site.

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CHAPTER TWO

GREENHOUSE STUDY: EFFECT OF ECTOMYCORRHIZAL COLONIZATION ON WHITEBARK PINE SEEDLINGS PLANTED IN SOIL FROM A BURN SITE

Introduction

Whitebark pine (*Pinus albicaulis*) is a keystone species that is unique to high elevation ecosystems in western North America (Arno and Hoff 1989), and in the northern Rocky Mountains whitebark pine forests cover approximately 10-15% of the forested landscape (Arno 1986, Tomback et al. 2001). In these forests whitebark pine occurs either as the dominant tree species in climax treeline communities or mixed with other conifers in seral stands typically at lower elevations (Arno and Hoff 1989). Whitebark pine is a five-needle pine and is the only stone pine native to North America (McCaughey and Schmidt 2001) and as such produces nutrient rich pine nuts (wingless seeds) in indehiscent cones (Lanner and Gilbert 1994) that are a major part of the diet of grizzly and black bears, squirrels, and other small mammals (Hutchins and Lanner 1982, Fortin 2011). Other ecosystem services provided by whitebark pine include regulation of snow melt (Farnes 1990), reduced erosion at certain high elevation sites (Weaver 2001), and facilitation of tree island formation at treeline (Resler and Tomback 2008, Tomback et al. 2014).

Wildfire plays an important role in the functioning of these unique whitebark pine habitats in the northern Rocky Mountains. In seral whitebark pine stands, fire serves as a

regenerative force, periodically clearing the area of competition (Arno and Hoff 1989). Whitebark pine seeds are dispersed by birds, mainly the Clark's nutcracker, which is capable of travelling long distances and often prefers to cache seeds on recent burn sites which offer large visual cues and microsites; this form of dispersal gives whitebark pine an advantage over wind-dispersed conifer seed for early establishment of recently burned areas (Tomback 1982, McCaughey 1990, Tomback et al. 1993, Lorenz et al. 2008). In climax whitebark communities where fire return intervals are longer, wildfire plays less of a role (Arno 2001).

The recent decline of whitebark pine is nearly range-wide due to several interacting threats that include invasive white pine blister rust (WPBR) (*Cronartium ribicola* Fisch.), native mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins), climate change, and fire suppression (Keane et al. 2012). Introduced to North America in the early 1900s (McDonald and Hoff 2001), WPBR is a non-native fungal pathogen with a complicated life-cycle that alternates mainly between white pines and *Ribes* spp. (Grossulariaceae), though other less common telial hosts include *Castilleja* and *Pedicularis* (Orobanchaceae; McDonald et al. 2006). The range of WPBR nearly encompasses that of whitebark pine (Schwandt et al. 2010) and some areas have up to 100% infection rates (Keane et al. 2012). Although the MPB is native to North America, recent outbreaks have been severe and it was estimated that almost half a million acres of whitebark pine were killed in 2007 (Gibson et al. 2008). Whitebark pine trees infected with WPBR are more susceptible to MPB attacks (Bockino and Tinker 2012) and a generally warming climate has potentially exacerbated the spread of both (Koteen 2002,

Logan et al. 2010). Lastly, fire suppression in the 20th century has increased fire return intervals, resulting in a decrease of seral whitebark pine stands that rely on periodic wildfire to reduce competition with other more shade-tolerant species (Arno 2001).

Whitebark Pine and Ectomycorrhizal Fungi

The vast majority of vascular plants, including whitebark pine and other pines, rely on mycorrhizal associations for the uptake up nutrients (Smith and Read 2008, Hobbie and Högberg 2012). In this mutualism, the fungus provides increased nutrient uptake via an expanded surface area of the roots through a large mycelial network as well as enzymatic capabilities that allow access to different forms of nutrients otherwise unattainable by the plant (Brundrett et al. 1996, Hobbie and Högberg 2012). In return the fungus receives photosynthetically derived carbohydrates from the plant (Allen et al. 2003). In particular, most woody plants associate with ectomycorrhizal (ECM) fungi, that form an external fungal layer called a mantle over fine root tips; hyphae grow between cortical root cells forming what is called a Hartig net which serves as the interface of nutrient exchange between plant and fungus (Brundrett et al. 1996, Smith and Read 2008).

Whitebark pine forests exist in extreme, harsh, high elevation habitats and consequently the diversity of ectomycorrhizal fungi may be limited. The only study to survey whitebark pine forests across the Greater Yellowstone Ecosystem revealed fewer than 50 species of ECM fungal associates (Mohatt et al. 2008), while Douglas fir is known to host over 2,000 species (Trappe 1962). Of the species identified, the suilloid clade (Bruns et al. 1989), comprised primarily of the genera *Suillus* and *Rhizopogon*, is of

marked importance to whitebark pine in the GYE (Mohatt et al. 2008). The genera *Suillus* and *Rhizopogon* are closely related and some of their species have shown high specificity for certain hosts (Grubisha 1998). A limited number of *Suillus* species are specific for five-needle pines or stone pines such as whitebark pine (Bruns et al. 2002, Grubisha et al. 2002, Cripps and Antibus 2011). *Rhizopogon* species are also somewhat host restricted, but are not typically tied to a particular tree species (Bruns et al. 2002).

Worldwide, out of over 100 total species of *Suillus*, around 20 occur with 5-needle pines (Klofac 2013). Thirteen of this subset occur in western North America. Only three of these have been recorded in the northern Rocky Mountains with whitebark pine and this includes: *Suillus sibiricus* (Singer) Singer (= *S. americanus* (Peck) Snell), *Suillus subalpinus* M.M. Moser, and *Suillus discolor* (A.H. Sm, Thiers & O.K. Miller) N.H. Nguyen (Mohatt et al. 2008, Cripps and Antibus 2011, Nguyen et al. 2016). Of these, *S. sibiricus* is also known to occur with stone pines in the Swiss Alps and the Altai mountains in central Asia (Moser 2004), and with at least the five-needle pine species *P. monticola*, *P. flexilis*, and *P. strobus* in North America. *Suillus discolor* appears restricted to *P. albicaulis*, *P. flexilis*, and *P. monticola* in North America, while *S. subalpinus* has only been found with whitebark pine (Klofac 2013, Nguyen et al. 2016).

The association of *Suillus* to pines, and thus to whitebark pine, appears to be ecologically important. First, there is evidence of a long co-evolutionary history between particular *Suillus* and *Pinus* species (Wu et al. 2000) and certain species such as *S. sibiricus* are found with stone pines worldwide (Moser 2004). Secondly, suilloid fungi including *Suillus* species appear to be capable of associating with both young seedlings

and mature trees of whitebark pine (Mohatt 2008, Trusty and Cripps 2011). Third, suilloid fungi may have a unique niche in the uptake of nutrients for whitebark pine (Cripps and Antibus 2011).

Suilloid Fungi and Fire

Generally, ECM fungal diversity decreases after wildfire, though this appears dependent on the species present as well as on the severity of the fire (Smith et al. 2005, Cairney and Bastias 2007). In the suilloid clade, *Rhizopogon* species were somewhat resistant to wildfire in a natural *P. muricata* forest; because sampling occurred shortly after the fire, researchers hypothesized that the fungi persisted through resistant spores rather than re-introduction through animal vectors (Baar et al. 1999). Other studies in which heat treatments were applied to soils containing fungal spores found increased viability in some *Rhizopogon* species but reduced spore viability in *Suillus pungens* Thiers & A.H. Sm. (Izzo et al. 2006, Peay et al. 2009). It may be that *Suillus* in general has low resistance to wildfire. Another study found that *S. pungens* genets from a recently burned *P. muricata* forest did not match the genets present before the fire, which indicates that the species may not have survived the fire but was instead reintroduced by wind-dispersed spores from neighboring populations (Bruns et al. 2002). In a greenhouse study that compared the ectomycorrhizal colonization of *P. muricata* seedlings planted in pre-fire and post-fire soil (one month after burning) from *P. ponderosa* forests, researchers found that two species of *Suillus* decreased in occurrence in the post-fire soil (Glassman et al. 2015). Jones et al. (2010) found that after low severity burns of Douglas-fir forests, *Suillus* and *Rhizopogon* species made up about 9% of the relative abundance

of the ECM fungal community, but after high severity burns these species were at 0% relative abundance (absent).

Trusty and Cripps (2011) compared the ectomycorrhizal community on roots of whitebark pine seedlings regenerating in a severely burned forest 5 years post-fire to those of seedlings in an adjacent unburned forest. Overall there was a 40-60% reduction in ECM diversity and a 10-13% reduction in suilloid fungi on seedlings at the burned site; the presence of at least low levels of suilloid fungi at the burned site was attributed to the presence of animal vectors and a convenient source of inoculum (mature whitebark pine trees) in the adjacent unburned forest. In summary, it appears that recolonization by *Suillus* species after a severe fire can potentially take several years and most likely occurs through wind and animal dispersed spores from adjacent forests and not through survival of fungal propagules in soil, at least on severe burns. Additionally, the drastic decline of whitebark pine throughout its range also means a reduction in healthy sources of inoculum.

Restoration of Whitebark Pine

Growing concerns over the widespread decline of whitebark pine have spurred a concerted effort between researchers, land managers, and the public to protect and restore this iconic species across its range. The main strategy for restoration is centered on the outplanting of blister rust resistant seedlings in areas where whitebark pine has experienced the highest mortality rates (Keane et al. 2012). This time-consuming process includes the identification of phenotypically resistant “plus” trees in the field, protection and collection of seed, constant development of nursery practices for raising seedlings,

multiple rounds of screening progeny for inherited resistance, establishment of seed orchards, and ultimately the outplanting of resistant lines of seedlings in established seed zones (Mahalovich and Dickerson 2004, Keane et al. 2012). Because this process is so demanding, it is important to refine and fully understand each step in the development of an effective and efficient restoration program.

Efforts to monitor the survival of outplanted whitebark pine seedlings over the last decade have revealed that despite these intense efforts, long term survival rates are low. In a large scale project, Izlar (2007) monitored over 114,000 seedlings across 48 planting sites and found that while first year seedling survival rates were high (74%), 3rd-15th year survival rates were much lower (38%); in some areas, no seedlings survived. Land managers are constantly working to refine nursery and planting practices in the interest of increasing these survival rates, but it is also important to understand factors that may influence the success of seedlings once they are outplanted.

Current guidelines recommend planting seedlings with microsites (shelter objects such as logs and stumps) in areas where there is adequate moisture and reduced competition (McCaughey et al. 2009). Land managers often choose burn sites previously dominated by deteriorating populations of whitebark pine as they provide areas clear of competition and because there may not be a sufficiently healthy seed source nearby to initiate natural regeneration (Keane et al. 2012, Leirfallom 2014). Leirfallom (2014) found that natural regeneration of whitebark pine at burn sites was low if <50% of mature trees in adjacent stands were healthy. Studies have shown that outplanting on burn sites can potentially increase seedling survival rates (Izlar 2007, Asebrook and Hintz 2015);

one study found increased germination and survival after direct planting of whitebark pine seed in burns (Perkins 2015).

After intense wildfire, a "pulse" of inorganic N, mainly in the form of ammonium (NH_4^+), is immediately released through combustion followed by subsequent nitrification of ammonium into nitrate (NO_3^-) (Covington and Sackett 1992, Certini 2005, Delwiche 2010). If seedlings are planted within a few years following a severe wildfire, this pulse of nutrients could likely benefit seedlings in the critical establishment period (Bansal et al. 2014). This could be especially true in subalpine forests where nitrogen is often a limiting factor (Körner 2003). Perkins (2015) directly outplanted whitebark pine seedlings on adjacent burned and unburned forest sites and reported that seedlings planted on the burned sites not only had higher survival rates and faster growth in comparison to seedlings planted on unburned sites, but also increased foliar nitrogen. Soil testing done 2-3 years after a severe prescribed fire at one site revealed that soil from the burned area contained more than three times the nitrate than soil from the adjacent unburned area (Perkins 2015). However, if not utilized soon after, nitrate is likely to be leached downwards in the soil and the ammonium is either adsorbed by the soil or transformed through nitrification (Certini 2005).

Ectomycorrhizal Fungi in Restoration

Current practices for generating whitebark pine seedlings in the United States typically do not include the inoculation of seedlings with ectomycorrhizal fungi before outplanting, although this process is being initiated on a large scale in Canada and it is recommended for severe burns (Keane et al. 2012). Host specific fungi have been used in

the successful restoration of other pine species. For example, *Suillus* species have been used in Austria in the successful restoration of the European stone pine (*Pinus cembra* L.), a close relative of whitebark pine; inoculation prior to outplanting increased survival rates 20-80% and this has been maintained for approximately 30 years after planting (Moser 1956, Schmid 2006). Decades after planting, the original *Suillus* inoculants were colonizing 82% of the root tips sampled, indicating the sustainability of *Suillus* colonization (Rainer et al. 2015). *Rhizopogon* species are also commonly used as commercial inoculum in restoration practices (Amaranthus 2002), but they are not as host specific and have not been tested on a large scale for whitebark pine.

The selection of appropriate ectomycorrhizal fungi is a critical component in the restoration of particular hosts (Vosátka et al. 2008). Whitebark pine grows in very specific habitats and hosts a limited diversity of fungi (Mohatt et al. 2008), therefore general commercial fungal inoculum is not appropriate and risks adding alien fungi into a sensitive system (Schwartz et al. 2006, Keane et al. 2012). Inoculation trials at Montana State University screened over 25 strains of native ECM fungi associated with whitebark pine and identified *Suillus* species, particularly *S. sibiricus*, as vigorous colonizers of whitebark pine seedlings in the greenhouse; the less host-specific *Rhizopogon* species were not as effective as colonizers in the allotted time period (Cripps and Grimme 2011).

Inoculation of seedlings prior to outplanting can be especially critical when the planting area is likely devoid of a diverse population of ECM fungi; this applies to recently severely burned forests or whitebark pine ghost forests where the majority of mature trees have died (Cripps and Grimme 2011). If there is a nearby potential source of

inoculum such as a healthy, mature whitebark pine forest, wind or animal vectors may serve to reintroduce fungi to the area to be planted and inoculation may not be necessary (Ashkannejhad and Horton 2006, Trusty and Cripps 2011). However, re-establishing these fungal populations through natural dispersal can take time (Trusty and Cripps 2011), and inoculation of seedlings and establishment of ectomycorrhizae on the roots prior to outplanting may benefit seedlings in the critical establishment period immediately after planting (Ortega et al. 2004, Quoreshi et al. 2009). If inoculation with ECM is successful in sufficiently enhancing survival rates, it could provide a cost-effective way for land and resource managers to optimize seedling resources and improve upon out-planting success of whitebark pine seedlings.

Initial studies that utilized *Suillus* fungi to inoculate whitebark pine seedlings prior to outplanting have shown promising results. A recent study in Waterton Lakes National Park that examined various planting treatment combinations showed that seedling survival was improved when they were planted in prescribed burned areas, with microsites, and subjected to inoculation with *S. sibiricus*; approximately 11% of the increase in survival was directly attributable to inoculation alone (Cripps et al. 2014, Lonergan et al. 2014). In Glacier National Park, seedlings inoculated with *S. sibiricus* and outplanted on burn sites had higher survival rates in comparison to un-inoculated seedlings on these sites; additionally, preliminary data (though not statistically significant) suggests possible enhanced growth of inoculated seedlings in comparison to seedlings that were not inoculated (Asebrook and Hintz 2015). These studies were not on severe burns which are more likely to be devoid of ECM fungi. Also, neither assessed

actual successful root colonization after inoculation prior to outplanting as subsequent destructive sampling in the field was not possible. Inoculation experiments have shown that not all inoculated seedlings actually become colonized (Cripps and Grimme 2011). Therefore, using 'inoculation' as an explanatory 'treatment' to explore colonization may mask any benefits of actual root colonization. While studies have shown that inoculation of whitebark pine seedlings before they are planted on burn sites has the potential to increase overall seedling survival, not much is known about the actual physiological processes in seedlings.

Effects of Ectomycorrhizal Colonization on Seedlings in a Greenhouse Setting

Seedlings used for restoration are often assessed casually for morphological and physiological qualities that might indicate vigor on outplanting, although morphology is used more often for convenience (Haase 2008). Typical morphological measures include height, stem diameter, root and shoot biomass, shoot:root ratio, and color; physiological traits include cold hardiness, root growth potential (RGP), and micronutrient and macronutrient concentrations in plant tissue (Haase 2008). For whitebark pine, priorities are often focused on fertilizer and watering regimes in the interest of achieving vigorous seedlings that are greater in height or biomass due to lateral branching and stem diameter (Burr et al. 2001). Standard fertilizer regimes are not conducive to ectomycorrhizal colonization of whitebark pine, however application of a low nitrogen fertilizer allows maintenance of ECM colonization in the greenhouse (Cripps and Grimme 2011, Lonergan and Cripps 2013).

Certain ectomycorrhizal fungi, especially suilloids, play an important role in the uptake of nitrogen in woody plants. Foliar nitrogen of host plants is one measure of the general vigor of both colonized and uncolonized plants. Foliar nitrogen is measured as a concentration (%) or as total content (g or mg), which is calculated by multiplying foliar biomass and concentration (Haase 2008). Both measurements are commonly used but when assessing foliar nitrogen, the growth rate of the plant should be taken into account; fast growth can lower nutrient concentrations in needles while overall nutrient content is increased due to higher biomass (Kohzu et al. 2000). Colonization by ECM fungi can potentially create a carbon sink in the plant, at least initially; however, the carbon sink created by ECM colonization also has the potential to increase photosynthetic rates (Dosskey et al. 1990). Nutrient status can indicate plant physiological and metabolic processes as well as outplanting performance (Haase 2008). Many studies have shown that inoculation with ECM fungi can have a positive effect (increased biomass, growth, and foliar nitrogen content) on seedlings of various pine species in a greenhouse setting (Heumader 1992, Hobbie and Colpaert 2003, Rincón and Fernández-Pascual 2007, Verma et al. 2014). Results vary by fungus, tree species, substrate, and other variables. However, no studies have specifically examined how colonization by *Suillus* species affects whitebark pine seedlings parameters in the greenhouse, and in particular those planted in soil from a burn site to simulate outplanting.

Analyses of Isotopic Signatures for
Investigations of the Plant-Fungal Relationship

Isotope signatures of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) have been used to examine the mycorrhizal system in several ways. They have been measured in the sporocarps of macro-fungi to determine their ecological status; for example, mycorrhizal fungi have enriched $\delta^{15}N$ signatures in contrast to saprobes, while saprobes are comparably enriched in $\delta^{13}C$ (Hobbie et al. 2012). Isotopic signatures have also been measured in plants, primarily mature trees in the field, as tracers of plant functioning and nutrient cycling (Dawson et al. 2002). Mycorrhizal fungi alter foliar $\delta^{15}N$ in host plants through isotopic fractionation. In the assimilation of nitrogen, mycorrhizal fungi preferentially hold onto ^{15}N while passing compounds containing fractionated nitrogen (with proportionately more ^{14}N) to the host; therefore, seedlings colonized by mycorrhizal fungi have foliage that is $\delta^{15}N$ depleted in comparison to seedlings that are not colonized (Hobbie and Colpaert 2003). These fungal associations also can affect plant foliar $\delta^{15}N$ in natural systems by expanding plant access to previously unavailable sources of nitrogen such as ^{15}N-depleted surface litter or ^{15}N-enriched sources deeper in the soil (Hobbie and Högberg 2012). Different types of mycorrhizal associations can affect fractionation as well; Craine et al. (2009) compiled data on over 11,000 species of plants and showed that on average, non-mycorrhizal plants were the least depleted in foliar $\delta^{15}N$ (0.9±0.2‰), followed by hosts of arbuscular mycorrhizal fungi (-1.1±0.1‰), followed by hosts of ectomycorrhizal fungi (-2.3±0.2‰), and finally hosts of ericoid mycorrhizal fungi (-5.0±0.2‰). Both field studies and greenhouse studies have reported a

negative correlation between ectomycorrhizal colonization and foliar $\delta^{15}N$ of host plants, which in turn can provide useful insight into plant-fungal nutrient dynamics.

Suillus fungi are particularly involved in the uptake of nitrogen (Keller 1996) and are close associates of pines (Bruns et al. 2002, Grubisha et al. 2002, Cripps and Antibus 2011). Therefore, these fungi are prime candidates for exploring the effects of ECM colonization on foliar $\delta^{15}N$ of pines (*Pinus*) and its relationship to other parameters.

Foliar $\delta^{13}C$ values have also been correlated with ectomycorrhizal status but the implications of this relationship are less clear. Mycorrhizal fungi demand a large amount of carbon from their host plants and as such can create a carbon sink, at least temporarily, and an initial reduction in growth (Miller et al. 1989, Colpaert et al. 1996). Hobbie and Colpaert (2004) hypothesized that because ectomycorrhizae create a carbon sink which may result in an increase in photosynthesis in colonized host plants (Dosskey et al. 1990), plant foliar $\delta^{13}C$ will also increase due to drawdown of foliar carbohydrate concentrations. Therefore it can be useful to explore carbon allocation and carbon isotope ratios for mycorrhizal and non-mycorrhizal plants. Plant water use efficiency (WUE) is often correlated with foliar $\delta^{13}C$ but other factors such as the concentration of available nitrogen can affect WUE, and nursery plants are typically watered to saturation (Farquhar et al. 1989, Hobbie and Colpaert 2004).

The measurement of seedling parameters such as biomass and nutrient status along with the analysis of isotopic signatures can help elucidate physiological interactions between plant and fungus in this mutualistic relationship; results can suggest

how overall seedling vigor and ultimately survival might be affected. This has not been explored for whitebark pine.

Research Objectives

This greenhouse experiment complements the field study (Chapter 3) which examines how *Suillus* species already established on root systems affect the survival of whitebark pine seedlings when they are planted on a burn site for restoration purposes. Destructive sampling was not possible in the field, and this study fills the gap by taking a closer look at morphological and physiological parameters in the greenhouse that might help explain field results.

The main goal of this experiment is to investigate the effects of ectomycorrhizal colonization by *Suillus* fungi on whitebark pine seedlings planted in soil collected from a burn site (hereafter referred to as "burn soil"). The primary objectives of the research are to determine: 1) whether native ectomycorrhizal fungi are initially present in the burn soil; 2) whether *Suillus* species colonizing the roots of nursery seedlings are able to grow into the burn soil; and 3) how colonization by any of these ectomycorrhizal fungi affects seedling parameters such as height, stem diameter, biomass, and nutrients in needles. Isotope analysis was used to help confirm that any differences in parameters were due to ectomycorrhizal colonization. Isotopic signatures are commonly used to investigate the movement of compounds in natural systems, however very few studies have attempted to examine how ectomycorrhizal colonization affects isotopic signatures in a controlled setting.

Insight gained from this experiment has the potential to aid land managers in improving best planting practices for whitebark pine seedlings by improving seedling fitness on out-planting. It remains to be discovered how results will relate to ultimate seedling survival in the field.

Materials and Methods

Soil Collection and Analysis

The Gravelly Mountain Range of southwestern Montana lies in the Beaverhead-Deerlodge National Forest (BDNF) and is part of the Greater Yellowstone Ecosystem (GYE). The range is defined by high intermountain valleys and steep mountain slopes, ranging in elevation from approximately 5,500 to 10,500 ft. (1,600-3,200 m) with treeline occurring around 9,500 ft. (2,900 m; Cooper et al. 1997, Hamlin and Ross 2002). Upper subalpine areas support pure stands of *Pinus albicaulis* (whitebark pine) as well as mixed stands of *Picea engelmannii* and *Abies lasiocarpa* (Cooper et al. 1997). The soil type is a gravelly loam derived from limestone, sandstone, and shale (Soil Survey Staff, USDA Web Soil Survey). Over the past few decades whitebark pine has greatly declined across the BDNF largely due to the mountain pine beetle and white pine blister rust (GYCC 2011).

In 2013, the Eureka Basin fire burned over 6,000 acres in the Gravelly Mountains and was classified as high intensity fire resulting in a moderate to high severity burn; much of the area burned was mixed whitebark pine stands with a significant presence of dead whitebark pine snags (BDNF 2013, Olson 2015). A 107-acre whitebark pine

planting unit was designated by the BDNF to be planted with whitebark pine as part of a 10-year restoration plan (Olson 2015). The planting unit was severely burned with only dead standing cover and minimal regeneration after two years (Figure 2.1). Soil was collected from the planting unit (referred to as "burn soil") for the greenhouse bioassay in 2015, approximately two years post-fire.

Figure 2.1: Severely burned planting unit in the Eureka Burn two years post-fire.

Approximately 75 liters of soil was collected along two 50 meter transects representative of the planting unit. For each transect, 3-4 liters of soil was collected every 5 meters to a depth of 15-20 cm. Samples were mixed and transported to the Montana State University Plant Growth Center (PGC) and placed in cold storage. A subsample was sent to Agvise Laboratories for an elemental analysis (Table 2.1). The burn soil had a pH of 5.9, a cation exchange capacity (CEC) of 18.9 meq, organic matter content of 11.8%, and the texture is a coarse loam.

Seedlings

Approximately 140 whitebark pine seedlings were obtained for the experiment from the Coeur d'Alene Forest Service Nursery in Idaho. Seedlings were one and a half years old and had been grown from seed collected in September 2003 in the Bridger-Teton National Forest at Moccasin Basin, WY, which lies in the Greater Yellowstone/Grand Tetons (GYGT) seed zone (Mahalovich et al. 2006). Seedlings were sown at the nursery by placing stratified non-scarified seeds in Ray Leach SC10 Super Cone-tainers (3.8 cm diameter, 21 cm depth; Stuewe & Sons, Inc., Tangent, Oregon) in a media of ground Canadian Sphagnum peat moss and composted Douglas fir bark (7:3). Seedlings were maintained for two growing seasons under standard nursery practices that included fertilization once or twice a week with $20N:7P_2O_5:10K_2O$ fertilizer (Peter's Professional, The Scotts Company, Marysville, Ohio) and STEM micronutrients (soluble trace element mix, Peter's Professional; Eggleston 2010, Robertson 2015). Seedlings were finished with a $4N:25P_2O_5:15K_2O$ fertilizer and overwintered in freezer storage for vernalization from December 2014 to May 2015 (Robertson 2015). At the time of this experiment the Coeur d'Alene Forest Service Nursery typically planted two stratified seedlings per container to ensure germination, and consequently each container held two seedlings.

Ectomycorrhizal Colonization of Seedlings:
Initial Assessment and Molecular Identification

Upon arrival at the Montana State University (MSU) Plant Growth Center (PGC), all root systems were visually inspected for ectomycorrhizal colonization, and seedlings

were separated into colonized and uncolonized groups based on the presence or absence of ectomycorrhizal root tips. These groups are subsequently referred to as "core-colonized" and "core-uncolonized" seedlings. The single dominant morphotype present appeared to be suilloid, characterized by dingy white "hand like" branching mycorrhizae, white rhizomorphs, and a lack of hyphal clamps (Treu 1990). To identify the fungi present, a total of 18 ectomycorrhizal root tips representative of the dominant morphotype were sampled for direct sequencing of the internal transcribed spacer (ITS) region of the fungal ribosomal DNA (Menkis et al. 2005).

For sampling, individual mycorrhizae were removed from the root systems of seedlings with sterilized fine tweezers, rinsed free of soil with deionized water, and placed directly in 1.5 mL screw-cap microcentrifuge tubes with 2-3 glass beads. Samples were disrupted in a MiniBeadbeater™ (BioSpec Products, Inc.) for two 30-second cycles on the lowest speed. DNA was extracted following the Quick-Start Protocol for the DNeasy® Plant Mini Kit (Qiagen) with the single exception of adding 50 µl of Buffer AE for the final elution instead of the recommended 100 µl in order to increase the final concentration of DNA.

For PCR amplification the following was added to a PCR tube for each sample: 2.0 µl mycorrhizal DNA, 12.5 RedTaq® ReadyMix™ (Sigma-Aldrich Co.), 1.0 µl forward primer (10 µM ITS1-F, IDT®), 1.0 µl reverse primer (10 µM ITS4, IDT®), and 8.5 µl molecular grade water (Gardes and Bruns 1996, Ola et al. 1999). Primers were chosen for fungal specificity (White et al 1990, Gardes and Bruns 1993). PCR tubes were placed in a Thermocycler for amplification of the ITS region running the following program: 94 °C

for 2 minutes followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 1 minute, and 72 °C for 1 minute, followed by a final 5 minute elongation step at 72 °C (Barge 2015). To determine whether PCR was successful, 10 µl of PCR product for each sample was loaded onto a 1.5% agarose gel with 0.0003% ethidium bromide and run at 90 volts for approximately one hour and then viewed with a trans-illuminator (Ola et al. 1999). For successfully amplified samples, PCR purification was performed using a QIAquick® Kit (Qiagen). Manufacturer's instructions were followed utilizing 30 µl of molecular grade water for the final elution for increased concentration of DNA. After PCR purification, the DNA concentration of each sample was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific).

For submission to the DNA Sequencing Facility at the University of California, Berkeley (http://mcb.berkeley.edu/barker/dnaseq/home), reactions were mixed for each sample separately for forward and reverse primers and brought to a total volume of 13 µl at a concentration of 100ng/1000bp DNA and 0.8 pmol/µl primer. Raw forward and reverse sequences received from UC Berkeley were aligned and edited using SeqTrace v. 0.9.0 (Stucky 2012). Edited sequences were compared to sequences in GenBank for best species matches using NCBI (National Center for Biotechnology Information) Standard Nucleotide BLAST (Basic Local Alignment Search Tool) (blast.ncbi.nlm.nih.gov).

Soil Planting Treatments

Burn soil was mixed and large rocks and root material were sifted out; smaller root material was replaced to serve as a potential source of fungal inoculum. As a control, half of the burn soil was spread to approximately 3 cm thick in bins and sterilized in an

autoclave on a 60-minute solid materials cycle. For improved drainage, sterilized and unsterilized burn soils were each diluted with equal parts Soil Mix 3 in a large electric barrel mixer. Soil Mix 3 consists of pasteurized MSU mix, vermiculite, and sifted Canadian Sphagnum peat moss (2:2:1) and has an average pH of 5.66. The pasteurized MSU mix consists of loam, peat moss, and washed concrete sand (1:1:1) with AquaGro 2000 G wetting agent blended in at a rate of 0.59 kg/m^3 (1 lb/yd^3) steam pasteurized at 70°C for 60 minutes (Lonergan and Cripps 2013). MSU mix is periodically analyzed and results from the time of the experiment were used to estimate the nutrient status of Soil Mix 3 (Table 2.1).

Table 2.1. Soil analysis for burn soil and estimates for Soil Mix 3 in parts per million (ppm) excluding pH. Mixed 50:50 for soil treatments 1, 2, and 3.

	Nitrate-N	Phosphorus	Potassium	Chloride	Sulfur	Boron	Zinc
Burn Soil	12	16	317	2.5	5	0.4	2.71
Soil Mix 3	15.2	(PO_4) 13.2	233	NA	(SO_4) 6.4	0.16	3.2
	Iron	Manganese	Copper	Magnesium	Calcium	Sodium	pH
Burn Soil	89.3	81.7	0.51	343	3,028	22	5.9
Soil Mix 3	34.4	12.8	0.4	22.8	60.8	36.4	5.7

Nitrate-N availability for the 50:50 mixture of burn soil and Soil Mix 3 was estimated by averaging the nitrate values (ppm) of soils by proportion. Burn soil nitrate was measured as 12 ppm and for Soil Mix 3 was calculated as 15.2 ppm, therefore the 50:50 mix is estimated to be 13.6 ppm (equivalent to mg kg^{-1}). Peat moss has negligible nutritional value but a high CEC, therefore the addition of Soil Mix 3 likely increased buffering capacity.

Core-colonized and core-uncolonized seedlings were transplanted from original nursery containers into D40 Large Deepots (6.4 cm diameter, 25.4 cm depth; Stuewe & Sons, Inc., Tangent, Oregon) with soil mixture treatments. Soil treatments are shown in Table 2.2 and are as follows: (1) 50:50 mixture of sterilized burn soil and Soil Mix 3 and (2) 50:50 mixture of unsterilized burn soil and Soil Mix 3. Due to concerns that burn soil might not be conducive to ectomycorrhizal colonization, an additional soil treatment was applied that consisted of (3) 100% Soil Mix 3, which is known to be conducive to ECM colonization (Lonergan and Cripps 2013). The final treatment (4) was a 50:50 mixture of unsterilized burn soil and Soil Mix 3, with seedlings dipped in a *Suillus sibiricus* spore slurry prior to planting; this treatment was used to determine whether the spore inoculum was viable in burn soil for applied purposes. Sporocarps used to make the spore slurry were collected from whitebark pine forests at approximately 9,000 ft. elevation in the Tobacco Root Mountains, MT in 2015. Slurry was made by grinding the hymenium of sporocarps for approximately 1 minute in a coffee grinder and straining the material into sterile distilled water. A hemocytometer was used to estimate spore concentration of the slurry which was then diluted to 1×10^6 spores/ml (Cripps and Grimme 2011). Seedling root systems were completely submerged in slurry before planting.

Soil treatments 3 & 4 were not applied for the purpose of direct comparison with treatments 1 & 2 and were not included in statistical analyses of seedling parameters. Included in Table 2.2 are abbreviations for soil treatments as they are referred to hereafter. Seedlings were grown for approximately 6 months in the PGC greenhouse

under standard conditions of 22 °C day and 18 °C night with a 16-hour photoperiod. Seedlings were watered to saturation 3 times a week and no fertilizer was applied.

Table 2.2. Soil planting treatments and number of replicates of core-uncolonized and core-colonized seedlings.

Core-uncolonized	Core-colonized	Soil Mixture	Soil Mixture Abbreviation
n = 17	n = 26	(1) 50:50 mixture of sterilized burn soil and Soil Mix 3	sterilized burn soil
n = 19	n = 20	(2) 50:50 mixture of unsterilized burn soil and Soil Mix 3	unsterilized burn soil
n = 8	n = 16	(3) 100% Soil Mix 3	soil mix 3
n = 13	n = 22	(4) 50:50 mixture of unsterilized burn soil and Soil Mix 3, dip inoculation	unsterilized burn soil, inoculated

Final Assessment of Mycorrhizal Colonization

After seedlings were grown for approximately 6 months in the various soil treatments, the new root growth into the burn soil was assessed for mycorrhizal colonization. The original inner core of each root system labeled ‘core-colonized’ or ‘core-uncolonized’ was not included in this assessment. It was not possible to separate the root systems of the two seedlings in each container and root systems were assessed together. Seedlings were removed from containers and all loose soil was washed from root systems. New roots growing in the treatment soil were easily distinguishable from the inner root-bound core formed in the original nursery containers (Figure 2.2).

Figure 2.2: New root growth as distinguished from core of inner roots. Left: Before soil was removed. Right: After soil was removed.

New root growth was separated by clipping with fine scissors and was gently washed with distilled water; roots were assessed under a dissecting microscope (Nikon SMZ 1500, Meridian Instrument Company, Inc., Kent, WA) for frequency and abundance of ectomycorrhizal colonization (Brundrett et al. 1996, Lonergan and Cripps 2013). For frequency (percent of seedlings colonized in each treatment), a seedling was considered 'colonized' if >1% of the new root growth was colonized (Marx and Cordell 1988). Abundance was assessed in two ways: 1) as a visual estimate of the percent of the root system colonized and 2) as the number of ectomycorrhizae present (Lonergan and Cripps 2013). Importance values were calculated by adding frequency and abundance percentages; this measure represents the overall significance of ectomycorrhizal colonization (Horton and Bruns 2001). Results were averaged for seedlings in each treatment.

The method of Agerer (1997) was used to describe general ectomycorrhizal morphotypes on seedling roots. There was essentially only one type present for all treatments which was characterized as a *Suillus* morphotype. *Suillus* ectomycorrhizae on whitebark pine roots are dingy white, with dichotomous ("hand-like"; Figure 2.3) to coralloid branching, a plectenchymatous mantle, white rhizomorphs, and hyphae lacking clamp connections (Treu 1990, Lonergan and Cripps 2013).

Figure 2.3: Left: A *Suillus* ectomycorrhiza on a whitebark pine seedling root tip. Right: Suilloid ectomycorrhizae colonizing whitebark pine seedling root systems.

Height and Stem Diameter

All seedlings were measured for height and stem diameter at the time of destructive harvesting. Because containers held two seedlings, height and stem diameter were measured and recorded for both seedlings. Height was measured from the cotyledon scar to the tip of the terminal bud (Haase 2008). Stem diameter was measured with a digital caliper just below the cotyledon scar (Mexal and Landis 1990, Haase 2008).

Seedling Biomass

Total seedling biomass was measured per container as it was not possible to separate the root systems of the two seedlings in each container. Shoots were separated from roots by cutting the stem at the point of root differentiation. Soil was removed from root-bound cores by gently washing and teasing apart roots. New root growth and old root cores were dried separately in the PGC plant drying room at 49 °C for approximately 3 days or until a stable dry weight was reached and recorded. Total root biomass and new root biomass were analyzed separately. Needles were removed from stems and dried separately at 49 °C until stable dry weights were recorded. Total shoot biomass represents the combined dry weight of needles and stems.

Foliar Nutrient and Isotope Assessment

Subsamples of dried needles were assessed for foliar nutrient content and were subjected to isotope analysis of ^{15}N and ^{13}C for soil treatments 1 (sterilized burn soil) and 2 (unsterilized burn soil). Dried needles of the two shoots (per container) were combined for the sampling procedure. Approximately 0.1 g of dried needles from each container was placed in a 1.5 ml screw cap microcentrifuge tube with 3 glass beads and ground to a powder by placing sample tubes in a tissue homogenizer for two 30 second cycles (Figure 2.4). Powdered samples were weighed on a 4 decimal place scale (Sartorius, Model BP210S, Sigma-Aldrich®) to 5-6 mg in 5x9 mm tin capsules (Costech Analytical Technologies, Inc., Valencia, CA) (Figure 2.4). Capsules were sealed using sterilized tweezers (Figure 2.4), placed in a plastic sample tray (Costech Analytical Technologies, Inc., Valencia, CA), and mailed with a sample submission form to the University of

California at Berkeley Center for Stable Isotope Biogeochemistry for processing (https://nature.berkeley.edu/stableisotopelab/). At the facility, samples were processed using a CHNOS Elemental Analyzer linked to an IsoPrime100 mass spectrometer which yields measurements of %N, δ^{15}N(‰), %C, and δ^{13}C(‰). Total container foliar nitrogen and carbon content were calculated by multiplying foliar %N or %C by total foliar dry weight (Landis et al. 2005). Ratios of foliar C:N were calculated by dividing total foliar carbon content by total foliar nitrogen content.

Figure 2.4: Left: Dried whitebark pine needles ground in screw cap tubes. Right: A sample packaged in a tin capsule.

Natural abundances of isotopes in a substance, represented as δ, are measured as a ratio relative to an internationally designated standard. Units of measurement are in parts per mil (‰) because deviations from the standard are so small. For example, ^{15}N is calculated using the following equation:

$$\delta^{15}N(‰) = (R_{sample} / R_{standard} - 1) * 1000$$

where R_{sample} represents the ratio of the heavier to lighter isotope, $^{15}N/^{14}N$ in this case, in the substance being sampled and $R_{standard}$ represents the ratio of the heavier to lighter isotope in the standard (Dawson et al. 2002, Hobbie and Högberg 2012). The standard for N is atmospheric N_2 which has an abundance ratio of 3.6765×10^{-3} (Dawson et al. 2002). For carbon, the standard has been designated by the International Atomic Energy Agency (IAEA) as "Vienna"-PDB which is a slight variation of the original standard of belemnite from the PeeDee formation (PDB) that is no longer available; the isotopic abundance ratio of $^{13}C/^{12}C$ for this standard is 1.1237×10^{-2} (Dawson et al. 2002). Therefore a positive value of δ indicates that the sample has more of the heavier isotope, or is enriched, in comparison to the standard and a negative value indicates that the sample is comparatively depleted.

Experimental Design

This is an observational study due to the non-random application of the "treatment" of ectomycorrhizal colonization. Seedlings from the Coeur d'Alene nursery were visually separated by initial conditions into core-uncolonized and core-colonized groups. Therefore, the experimental design is 2 core-colonization groups (colonized or not) x 2 soil treatments (sterilized and unsterilized burn soil). Soil treatments were randomly applied in the planting of seedlings and seedling trays were rotated monthly in the greenhouse. Containers were considered independent after accounting for soil treatment and initial colonization. The number of replicates in each treatment was limited by the availability of whitebark pine seedlings from the nursery (Table 2.2).

Statistical Analysis

Due to current planting practices at the Idaho Nursery, each container held two seedlings. For the analyses of height and stem diameter, the greater measurement of the two seedlings was used for each container. For the analyses of biomass, foliar nutrient content and isotope ratios, measurements are per container (two seedlings combined). Statistical analyses were performed utilizing R 3.1.2 statistical software (©2016 The R Foundation).

Over the course of the experiment a few seedlings that were initially designated as core-uncolonized were found to have new root colonization at the final mycorrhizal assessment (n = 10). This was most likely because colonization was minimal and overlooked at the initial assessment. These seedlings were excluded from statistical analyses.

Analyses for the response variables of seedling height, stem diameter, biomass, shoot:root ratio, foliar C:N ratio, foliar %N, and foliar %C were performed for seedlings in soil treatments 1 (sterilized burn soil) and 2 (unsterilized burn soil) by fitting standard regression models rather than by using analysis of variance (ANOVA) for the purpose of estimating specific contrasts of interest. The regression model for each response variable included initial colonization and soil treatment as the two main effects as well as a term for interaction between the two and is shown below:

$$y_{ijk} = \beta_i + \eta_j + \gamma_{ij} + \epsilon_{ijk}$$

where β_i represents the effect of initial colonization, η_j represents the effect of soil treatment, γ_{ij} represents the interaction between initial colonization and soil treatment,

and ϵ_{ijk} represents the random error associated with each container. Box plots and 95% confidence interval plots were utilized for data exploration. Boxplots provide exploratory information on the spread of the data while confidence interval plots provide a visual representation of statistical significance (Krzywinski and Altman 2014).

For the analysis of foliar nitrogen content and foliar $\delta^{15}N$ (‰), boxplots and scatterplots were used to explore the relationship between the two responses. In order to account for this relationship a full linear model was fit using a generalized least squares function in R which allows for a separate estimate of a negative correlation between two responses for the same container (rather than a linear mixed effects model which only allows for a positive relationship). The full model included an induced correlation structure between responses and a three-way interaction between the two main effects (initial colonization and soil treatment) and response type. After testing for interactions, a simplified final model was fit and is shown below.

$$y_i = \beta_0 + \beta_1 * I_{colB} + \beta_2 * I_{soil2} + \beta_3 * I_{N15} + \beta_4 * I_{colB}I_{N15} + b_{i[j]} + \epsilon_i$$

Before fitting the model, the responses of foliar nitrogen content and $\delta^{15}N$ were centered and rescaled on a standardized scale in order to account for the difference in units of measurement as the spread of both response variables are included in the estimation of residual standard deviation. Estimates for contrasts of interest were interpreted for total nitrogen content and foliar $\delta^{15}N$ after converting the estimates back to original units. To further analyze the relationship between foliar nitrogen content and $\delta^{15}N$, models were used to estimate the correlation coefficient which describes the direction and strength of the linear relationship between the two responses.

For the analysis of foliar carbon content and foliar $\delta^{13}C$(‰), boxplots and scatterplots were used to explore the relationship between the two responses. In order to account for this relationship a full linear model was fit using a generalized least squares function in R. The full model included an induced correlation structure between responses and a three-way interaction between the two main effects (initial colonization and soil treatment) and response type. Due to evidence of a three-way interaction, the full model was fit and is shown below.

$$y_i = \beta_0 + \beta_1 * I_{colB} + \beta_2 * I_{soil2} + \beta_3 * I_{C13} + \beta_4 * I_{colB}I_{C13} + \beta_5 * I_{soil2}I_{C13} + \beta_6 * I_{colB}I_{soil2}I_{C13} + b_{i[j]} + \epsilon_i$$

Before fitting the model, the responses of foliar carbon content and $\delta^{13}C$ were centered and rescaled on a standardized scale in order to account for the difference in units of measurement as the spread of both response variables are included in the estimation of residual standard deviation. Estimates for contrasts of interest were interpreted for total foliar carbon content and foliar $\delta^{13}C$ after converting the estimates back to original units. To further analyze the relationship between foliar carbon content and $\delta^{13}C$, models were used to estimate the correlation coefficient which describes the direction and strength of the linear relationship between the two response variables.

The number of ectomycorrhizal root tips on the new root growth of seedlings was visually related to foliar nitrogen content, $\delta^{15}N$, foliar carbon content, and $\delta^{13}C$ using scatterplots with smooth fitted trend lines.

Results

Molecular Analysis of Ectomycorrhizae

Of the total 18 ectomycorrhizal root tips sampled from the seedling core root systems for direct sequencing of the internal transcribed spacer (ITS) region, extraction of DNA and PCR amplification was successful in 9, as viewed on a gel with a trans-illuminator. Edited sequences were compared to sequences in GenBank for best species match. All matched at 99% identity with species in the genus *Suillus*; four matched *Suillus sibiricus* (Singer) Singer (= *S. americanus* (Peck) Snell) and 5 matched *Suillus discolor* (A.H. Sm, Thiers & O.K. Miller) N.H. Nguyen.

Assessment of Ectomycorrhizal Colonization

The mycorrhizal assessment showed that the *Suillus* species originally colonizing the core root systems of whitebark pine seedlings did grow onto the new roots in all soil treatments, indicating that the experimental burn soil was conducive to the growth of *Suillus*. The frequency of new root colonization of core-colonized seedlings ranged from 40-91% (Table 2.3). The abundance of new root colonization of core-colonized seedlings ranged from 12-28% with the lowest level in soil treatment 2 (unsterilized burn soil). Importance values for the new root colonization of core-colonized seedlings in all soil treatments ranged from 90-111% except for seedlings in treatment 2 (unsterilized burn soil) where the importance value was depressed to 50% (Figure 2.5).

In total, 10 of 57 seedling pairs in containers that were categorized as core-uncolonized exhibited colonization of new root growth (Table 2.3). For core-colonized

seedlings that exhibited new root colonization, frequency ranged from 0-32% and abundance ranged from 0-38%. Importance values for the new root colonization of core-uncolonized seedlings in all soil treatments ranged from 33-50%, however the number of replicates was low (Figure 2.5). It is hypothesized that at least some of these seedlings exhibited new root colonization due to low, undetectable levels of colonization at the initial mycorrhizal assessment. These 10 containers were removed from subsequent statistical analyses so as not to confound results.

Table 2.3. Average frequency and abundance of mycorrhizal colonization of new root growth into four soil types for core-colonized and core-uncolonized whitebark pine seedlings.

	Core-uncolonized*			Core-colonized		
Soil Treatment	Frequency	n	Abundance	Frequency	n	Abundance
(1) sterilized burn soil	12%	2	38%	73%	19	28%
(2) unsterilized burn soil	32%	6	18%	40%	8	12%
(3) soil mix 3	0%	0	0%	75%	12	15%
(4) unsterilized burn soil, inoculated	15%	2	18%	91%	20	20%

* Results for 10 seedlings that showed some colonization.

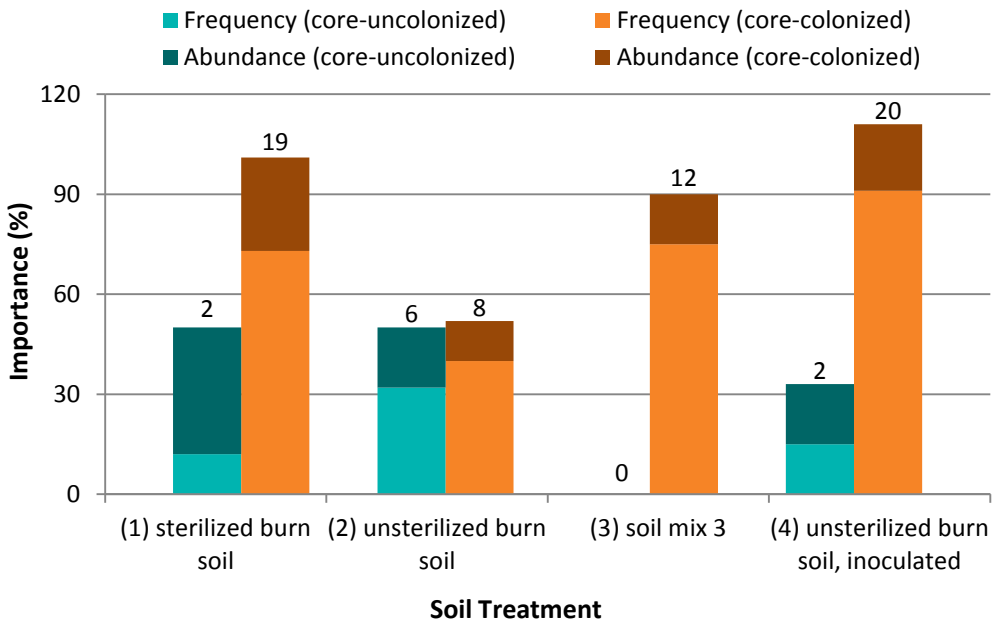

Figure 2.5: Frequency and abundance of ectomycorrhizal colonization of new root growth into four soil types for core-uncolonized and core-colonized whitebark pine seedlings.

The first objective of this bioassay was to determine whether a diversity of natural ectomycorrhizal fungi was present in the unsterilized burn soil by comparing results to that of sterilized burn soil. Over 99% of all ECM fungi observed in all soil treatments of this study were morphologically similar to the *Suillus* type. Both the frequency and abundance of new root colonization of core-colonized seedlings were greater in the sterilized burn soil (73% and 28% respectively) in comparison to colonization in unsterilized burn soil (40% and 12%) (Table 2.3).

In soil mix 3, 75% of core-colonized seedlings were colonized (frequency) at an average level of 15% (abundance) for the new roots, while there was no colonization of new roots for the core-uncolonized seedlings. In treatment 4 (inoculated), core-colonized

seedlings exhibited a greater frequency (91%) and abundance (20%) than core-uncolonized seedlings (15% and 18% respectively) (Table 2.3).

Height and Stem Diameter

For seedling parameters, only treatments 1 (sterilized burn soil) and 2 (unsterilized burn soil) were subsequently compared. In the analysis of seedling height and stem diameter, there was evidence of an interaction between core-colonization status and soil treatment; therefore differences in responses between core-colonized and core-uncolonized seedlings were estimated within each soil treatment. Data were explored using boxplots and confidence interval plots (Figure 2.6).

The average height of core-colonized seedlings planted in sterilized burn soil was 2.19 cm or 37% greater than that of core-uncolonized seedlings ($p = <0.001$). In unsterilized burn soil, there was no convincing evidence of a difference in height between core-colonized and core-uncolonized seedlings ($p = 0.33$); however, core-colonized seedlings tended to be slightly taller on average.

The average stem diameter of core-colonized seedlings planted in sterilized burn soil was 0.41 mm or 12% greater than that of core-uncolonized seedlings ($p = 0.013$). In unsterilized burn soil, there was no convincing evidence of a difference in stem diameter between core-colonized and core-uncolonized seedlings ($p = 0.84$). Averages and standard deviations are presented in Table 2.4; p-values are presented in Table 2.5

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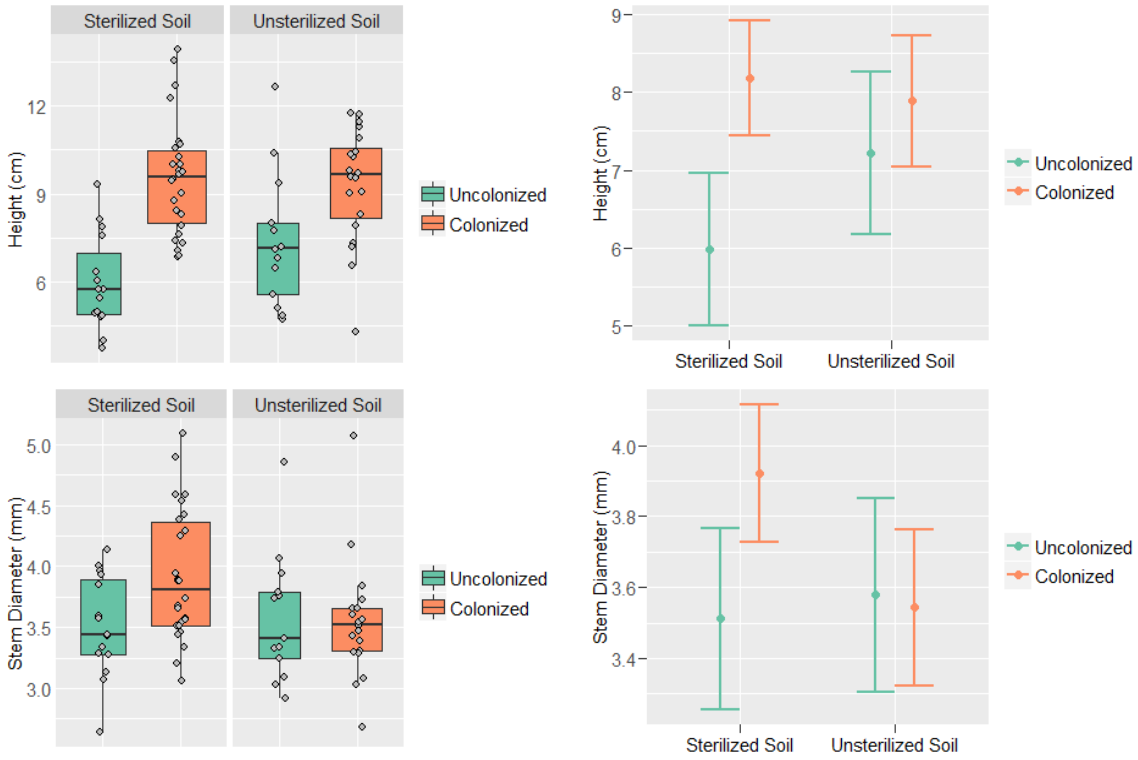

Figure 2.6: Boxplots and 95% confidence interval plots for height of whitebark pine seedlings by soil treatments 1 (sterilized burn soil) and 2 (unsterilized burn soil) and core-colonization status.

Seedling Biomass

For seedling parameters, only treatments 1 (sterilized burn soil) and 2 (unsterilized burn soil) were compared. In the analysis of total biomass, shoot biomass, and total root biomass of seedlings there was evidence of an interaction between core-colonization status and soil treatment; therefore differences in responses between colonized and uncolonized seedling pairs were estimated within each soil treatment. Data were explored using boxplots and confidence interval plots (Figure 2.7).

The average total biomass of core-colonized seedlings planted in sterilized burn soil was 3.6 g or 61% greater than that of core-uncolonized seedlings ($p = <0.001$). In unsterilized burn soil, the average total biomass of core-colonized seedlings was 1.95 g or

27% greater than that core-uncolonized seedlings ($p = 0.007$). The average shoot biomass of core-colonized seedlings planted in sterilized burn soil was 1.53 g or 63% greater than that of core-uncolonized seedlings ($p = <0.001$). In unsterilized burn soil, the average shoot biomass of core-colonized seedlings was 0.75 g or 24% greater than that of core-uncolonized seedlings ($p = 0.013$). The average total root biomass of core-colonized seedlings planted in sterilized burn soil was 2.06 g or 58% greater than that of core-uncolonized seedlings ($p = <0.001$). In unsterilized burn soil, the average total root biomass of core-colonized seedlings was 1.25 g or 29% greater than that of core-uncolonized seedlings ($p = 0.006$). Overall, core-colonized seedlings had a greater total biomass, shoot biomass, and total root biomass in comparison to core-uncolonized seedlings for both soil treatments. Averages and standard deviations are presented in Table 2.4; p-values are presented in Table 2.5.

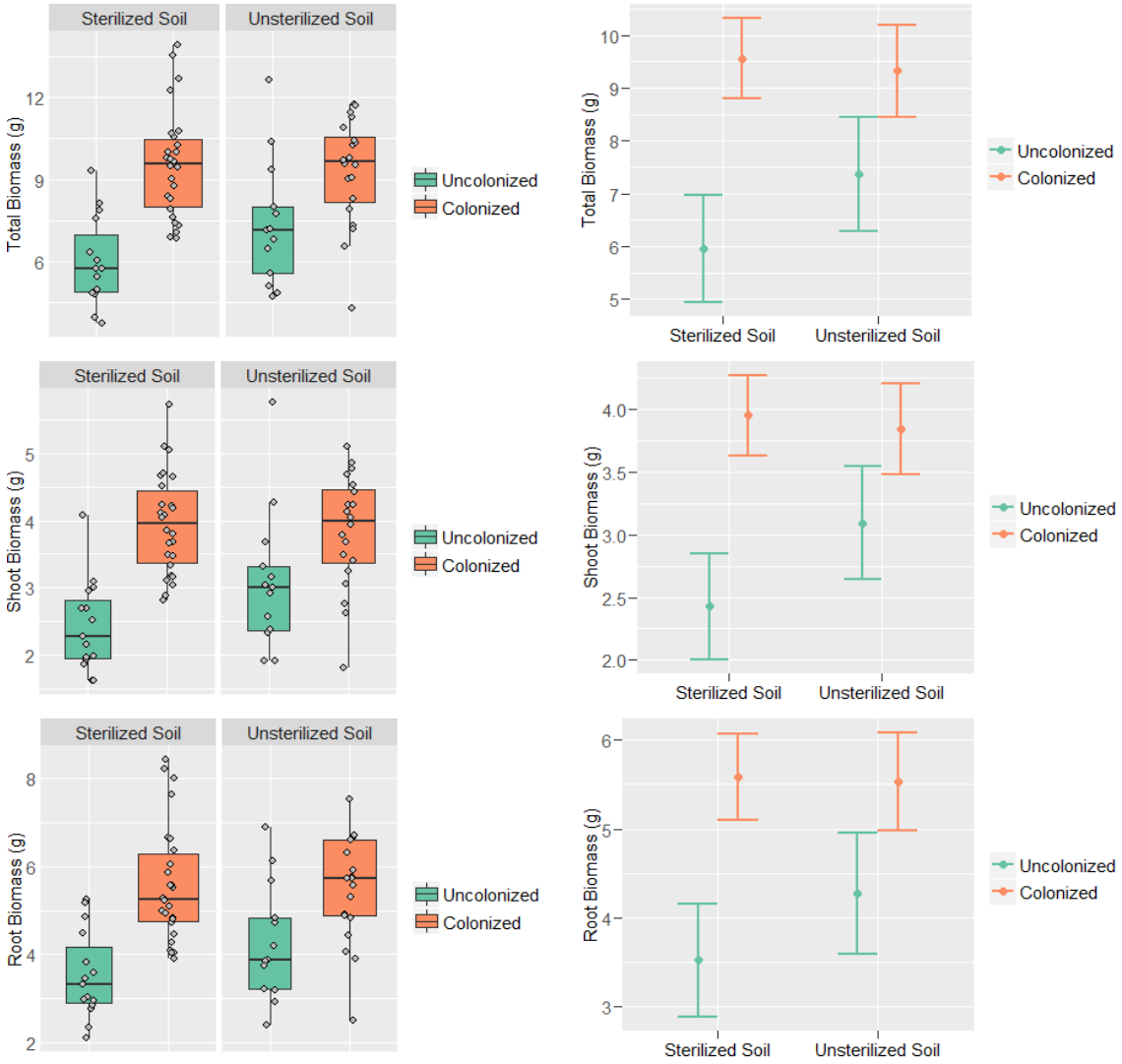

Figure 2.7: Boxplots and confidence interval plots of total biomass, shoot biomass, and root biomass of whitebark pine seedlings by soil treatments 1 (sterilized burn soil) and 2 (unsterilized burn soil) and core-colonization status.

In the analysis of shoot:total root ratio there was no evidence of an interaction between core-colonization status and burn soil treatment ($p = 0.28$); therefore the difference in response between core-colonized and core-uncolonized seedlings was averaged (pooled) across soil treatments. When averaged across soils, there was no evidence of a difference in shoot:total root ratio between core-colonized and core-

uncolonized seedlings (p = 0.98), suggesting that root and shoot growth remained proportional across treatments. Average total shoot:root ratios for all treatments ranged from 0.7 to 0.73 or approximately a 1:1.4 ratio (Table 2.4). Averages and standard deviations are presented in Table 2.4; p-values are presented in Table 2.5. The total needle biomass as a percent of total biomass per container is also included in Table 2.4 as an important measure for the discussion of isotope analysis.

New root biomass was analyzed in addition to total root biomass. Data were explored using boxplots and confidence interval plots (Figure 2.8). In the analysis of new root biomass there was no evidence of an interaction between core-colonization status and burn soil treatment (p = 0.87); therefore the difference in response between core-colonized and core-uncolonized containers was averaged (pooled) across soil treatments. The average new root biomass of core-colonized seedlings was 0.32 g or 21% greater than that of core-uncolonized seedlings averaged (pooled) across burn soil treatments (p = 0.008). Averages and standard deviations are presented in Table 2.4; p-values are presented in Table 2.5.

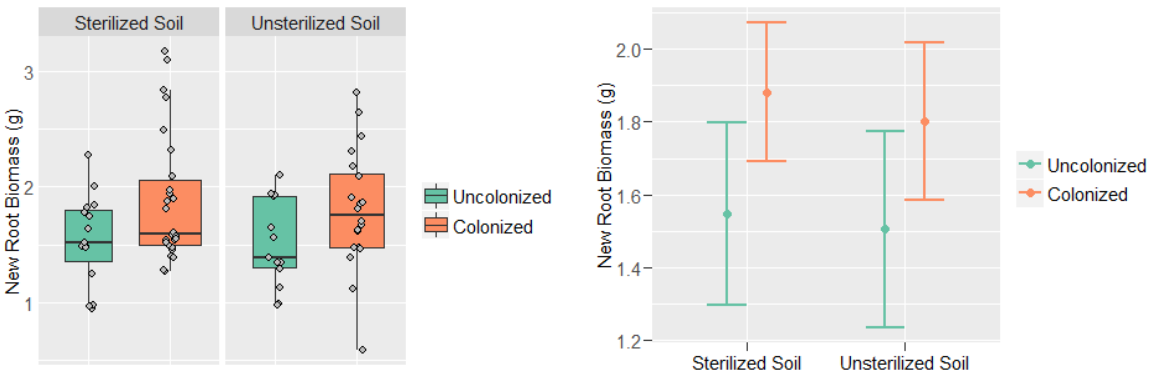

Figure 2.8: Boxplots and confidence interval plots of seedling new root biomass by soil treatment and core-colonization status.

Biomass ratios were also calculated for shoot:new root growth. In this analysis there was no convincing evidence of an interaction between core-colonization status and burn soil treatment ($p = 0.09$); therefore the shoot:new root ratios for core-colonized and core-uncolonized containers were averaged (pooled) across soil treatments. The average shoot:new root ratio of core-colonized seedlings was 0.37 or 20% greater than that of core-uncolonized seedlings averaged across burn soil treatments ($p = 0.007$), a result of proportionally more shoot than root growth in core-colonized seedlings. Averages and standard deviations are presented in Table 2.4; p-values are presented in Table 2.5.

Table 2.4. Mean and standard deviation for seedling parameters per container by core-colonization status and burn soil treatment.

	Colonization Status	Biomass (g)	Shoot Biomass (g)	Total Root Biomass (g)	Shoot: Root	New Root Biomass (g)	Shoot: New Root	Needle Biomass
Sterilized Burn Soil	core-uncolonized	5.95 ± 1.62	2.43 ± 0.67	3.53 ± 1.00	0.70 ± 0.10	1.55 ± 0.39	1.61 ± 0.43	25%
	core-colonized	9.55 ± 1.98	3.95 ± 0.75	5.58 ± 1.34	0.72 ± 0.10	1.88 ± 0.56	2.19 ± 0.47	27%
Unsterilized Burn Soil	core-uncolonized	7.37 ± 2.33	3.09 ± 1.04	4.28 ± 1.33	0.73 ± 0.11	1.51 ± 0.38	2.11 ± 0.74	28%
	colonized	9.32 ± 1.94	3.84 ± 0.85	5.53 ± 1.21	0.70 ± 0.09	1.80 ± 0.52	2.24 ± 0.55	26%

Table 2.5. Estimated differences, 95% confidence intervals, t-values, and significance for seedling parameters compared across core-colonized and core-uncolonized seedlings. Difference is positive if the parameter is greater for core-colonized seedlings.

Seedling Parameter	Treatment†	Estimated Difference, 95% CI (core-colonized vs core-uncolonized)	t-value	Significance
Height (cm)	sterilized burn soil	2.19 (0.96, 3.42)	3.55	**<0.001****
	unsterilized burn soil	0.66 (-0.69, 2.02)	0.98	0.33
Stem Diameter (mm)	sterilized burn soil	0.41 (0.09, 0.73)	2.54	**0.013***
	unsterilized burn soil	-0.04 (-0.39, 0.32)	-0.2	0.84
Total Biomass (g)	sterilized burn soil	3.6 (2.33, 4.87)	5.64	**<0.001****
	unsterilized burn soil	1.95 (0.55, 3.35)	2.78	**0.007***
Shoot Biomass (g)	sterilized burn soil	1.53 (1.00, 2.06)	5.73	**<0.001****
	unsterilized burn soil	0.75 (0.17, 1.33)	2.56	**0.013***
Total Root Biomass (g)	sterilized burn soil	2.06 (1.25, 2.86)	5.11	**<0.001****
	unsterilized burn soil	1.25 (0.37, 2.14)	2.83	**0.006***
New Root Biomass (g)	averaged across sterilized and unsterilized burn soil	0.32 (0.09, 0.55)	2.74	**0.008***
Shoot:Total Root Ratio	averaged across sterilized and unsterilized soil	-0.0006 (-0.05, 0.05)	-0.03	0.98
Shoot:New Root Ratio	averaged across sterilized and unsterilized soil	0.37 (0.11, 0.63)	2.79	**0.007***

Notes: Significant at * $P < 0.05$ and ** $P < 0.001$

† There is evidence of a significant interaction between soil treatment and core-colonization status ($p < 0.05$) for the first five parameters; therefore comparisons are displayed separately for each soil treatment. There is no interaction for the last three parameters which are averaged across soil treatments.

Foliar Concentration of Nitrogen and Carbon

In the analysis of foliar nitrogen concentration, there was an interaction between core-colonization status and burn soil treatment; therefore differences in responses between core-colonized and core-uncolonized seedlings were estimated within each soil treatment. The average foliar nitrogen concentration of core-uncolonized seedlings planted in sterilized burn soil was 22% greater than that of core-colonized seedlings ($p = <0.001$). In unsterilized burn soil, the average foliar nitrogen concentration of core-uncolonized seedlings was 9% greater than that of core-colonized seedlings ($p = 0.044$). Averages and standard deviations are presented in Table 2.6; p-values are presented in Table 2.7.

In the analysis of foliar carbon concentration, there was no evidence of an interaction between core-colonization status and burn soil treatment ($p = 0.5$); therefore the carbon concentrations for core-colonized and core-uncolonized seedlings were averaged (pooled) across soil treatments. When foliar carbon concentration for core-colonized and core-uncolonized seedlings was averaged across soil treatments, there was no convincing evidence of a difference ($p = 0.104$). Averages and standard deviations are presented in Table 2.6; p-values are presented in Table 2.7.

Foliar Nitrogen Content and Isotope Analysis ($\delta^{15}N$)

In the initial individual analyses of foliar nitrogen content and foliar $\delta^{15}N$, there was no evidence of an interaction between core-colonization status and burn soil treatment ($p > 0.05$); therefore differences in responses were averaged (pooled) across soil treatments. Core-colonized seedlings had significantly higher nitrogen content ($p <$

0.001) and were significantly depleted in foliar $\delta^{15}N$ ($p < 0.001$) in comparison to core-uncolonized seedlings.

Boxplots and scatterplots were utilized for exploration of the relationship between foliar nitrogen content and foliar $\delta^{15}N$ in seedlings (Figures 2.9 & 2.10). Plots indicate a negative relationship between foliar nitrogen content and foliar $\delta^{15}N$ in both core-colonized and core-uncolonized seedlings, though the relationship is weaker for core-colonized seedlings planted in sterilized soil.

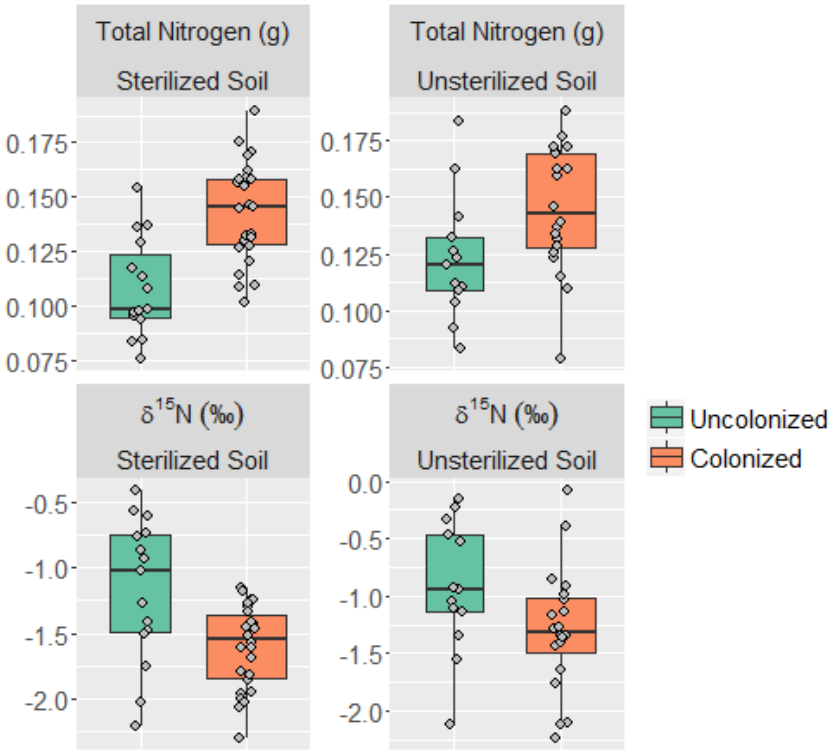

Figure 2.9: Boxplots of foliar nitrogen and foliar $\delta^{15}N$ (‰) as a response to burn soil treatment and core-colonization

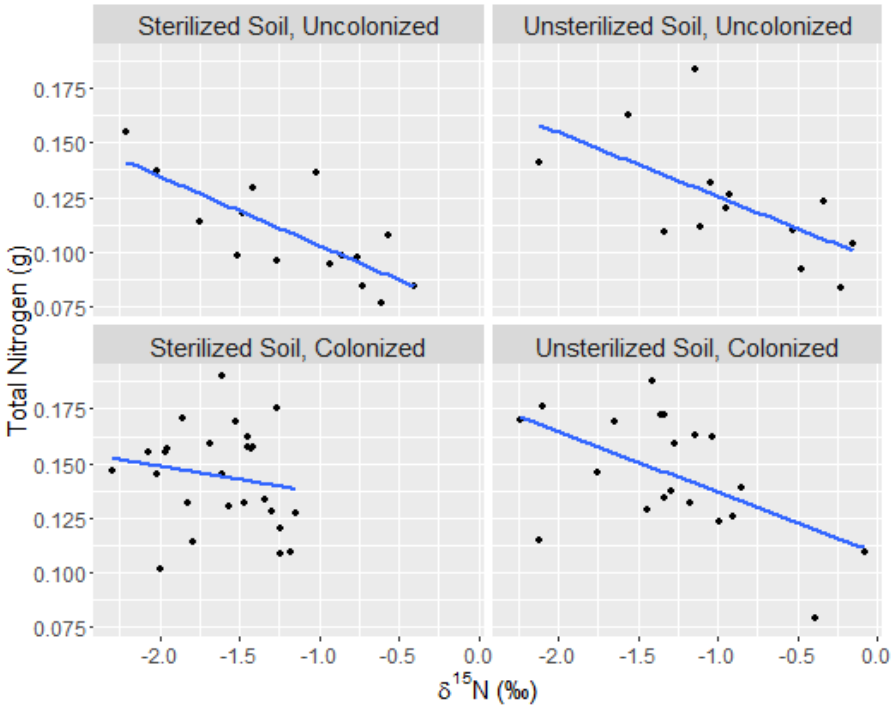

Figure 2.10: Scatterplots of foliar nitrogen content by foliar $\delta^{15}N$ (‰) for all treatment combinations.

Due to the apparent negative correlation between responses, a linear model was fit using generalized least squares. After fitting the full model there was no evidence of a 3-way interaction between burn soil treatment, core-colonization status, and type of response ($p = 0.44$). A second reduced model showed no evidence of an interaction between burn soil treatment and type of response ($p = 0.37$) or between burn soil treatment and core-colonization status ($p = 0.39$). A third reduced model did show strong evidence of an interaction between core-colonization status and type of response ($p = <0.001$) as indicated by the exploratory plots (Figures 2.9 & 2.10).

After the reduced model was fit, differences in foliar nitrogen content and $\delta^{15}N$ were estimated between core-colonized and core-uncolonized seedlings. As there was no evidence of an interaction between soil treatment and core-colonization status, estimates

were averaged (pooled) across sterilized and unsterilized soil. For interpretation, the standardized estimates were converted back to original units. In the analysis of total foliar nitrogen content, core-colonized seedlings on average contained 30 mg or 26% more foliar nitrogen than core-uncolonized seedlings when averaged across soil treatments ($p = <0.001$). In the analysis of foliar $\delta^{15}N$, core-colonized seedlings on average were depleted in $\delta^{15}N$ by 0.41‰ or 39% when compared to core-uncolonized seedlings when averaged across soil treatments ($p = <0.001$). Averages and standard deviations are presented in Table 2.6; p-values are presented in Table 2.7.

The estimated correlation coefficient for the relationship between foliar nitrogen content and $\delta^{15}N$ is -0.48 with a 95% confidence interval of -0.29 to -0.63. This coefficient indicates a negative linear relationship of weak to moderate strength.

Foliar Carbon Content and Isotope Analysis $\delta^{13}C$

In the initial individual analyses of foliar carbon content and foliar $\delta^{13}C$, there was no evidence of an interaction between core-colonization status and burn soil treatment ($p > 0.05$); therefore differences in responses were averaged across soil treatments. Core-colonized seedlings had significantly higher carbon content ($p < 0.001$) and were significantly enriched in foliar $\delta^{13}C$ ($p < 0.001$) in comparison to core-uncolonized seedlings.

Boxplots and scatterplots were utilized for exploration of the relationship between foliar carbon content and foliar $\delta^{13}C$ (Figures 2.11 & 2.12). The plots indicate a positive

relationship between foliar carbon content and foliar $\delta^{13}C$ for both colonized and uncolonized seedlings.

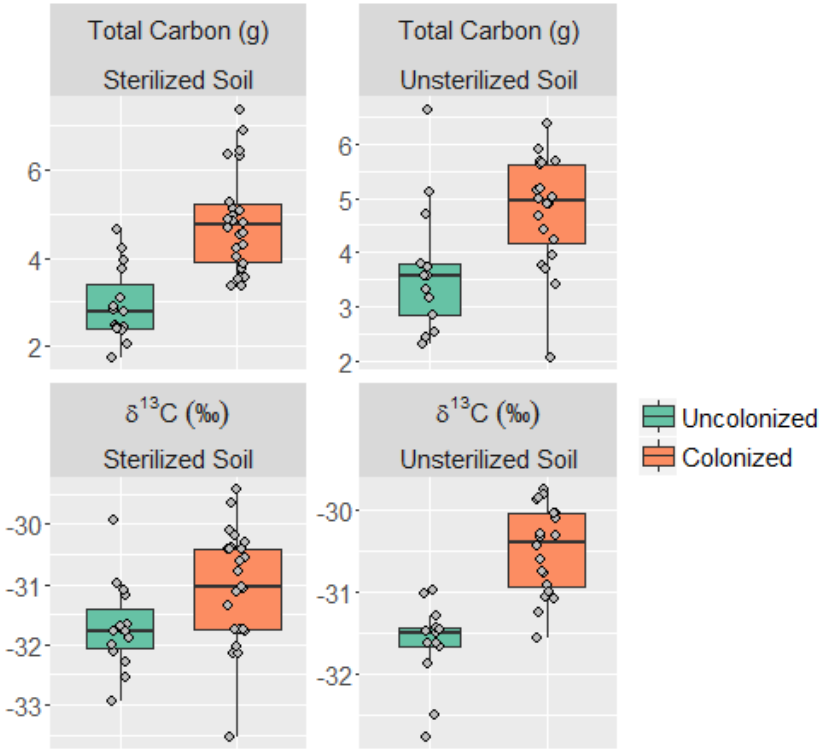

Figure 2.11: Boxplots of foliar carbon and foliar $\delta^{13}C$ (‰) as a response to burn soil treatment and core-colonization

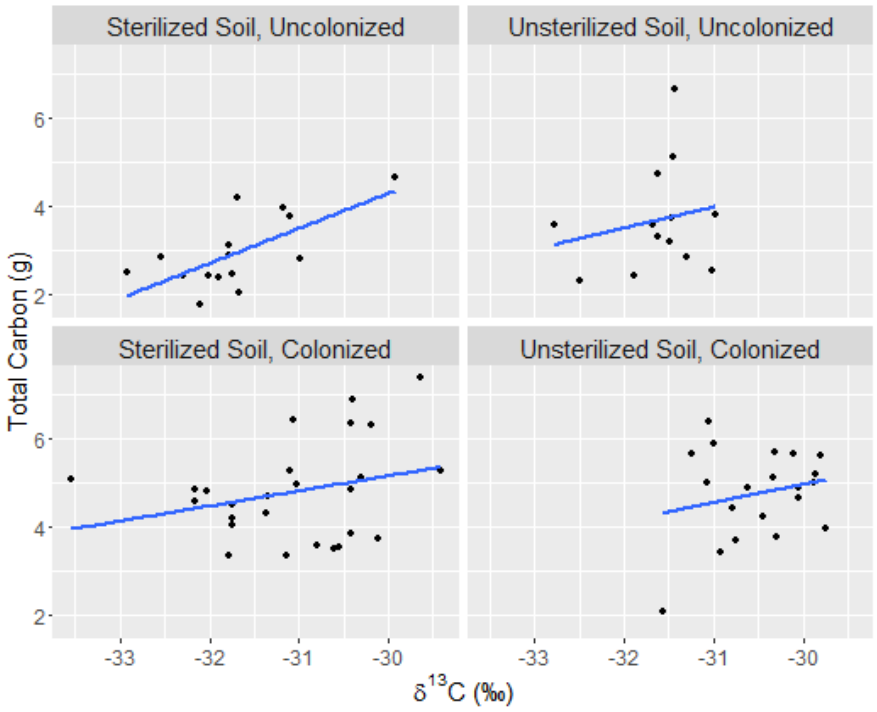

Figure 2.12: Scatterplots of foliar carbon content by foliar $\delta^{13}C$ (‰) for all treatment combinations.

Due to the apparent positive correlation between responses, a linear model was fit using generalized least squares. After fitting the full model there was evidence of a 3-way interaction between burn soil treatment, core-colonization status, and type of response ($p = 0.016$). Therefore, differences between core-colonized and core-uncolonized seedlings were estimated within each soil treatment for each response. For seedlings planted in sterilized burn soil, core-colonized seedlings on average had 1.86 g more or 63% greater carbon content than core-uncolonized seedlings ($p = <0.001$). In unsterilized burn soil, core-colonized seedlings on average had 1.09 g more or 30% greater carbon content than core-uncolonized seedlings ($p = 0.005$). For foliar $\delta^{13}C$(‰) of seedlings planted in sterilized burn soil, core-colonized seedlings on average were 0.66‰ enriched or 2%

greater than core-uncolonized seedlings ($p = 0.005$). In unsterilized burn soil, core-colonized seedlings on average were 1.14‰ enriched or 4% greater than core-uncolonized seedlings ($p = <0.001$). Averages and standard deviations are presented in Table 2.6; p-values are presented in Table 2.7.

The estimated correlation coefficient for the relationship between foliar carbon content and $\delta^{13}C$ is 0.31 with a 95% confidence interval of 0.085 to 0.503. This coefficient indicates a positive linear relationship of weak to moderate strength.

Foliar C:N

In the analysis of the foliar C:N (content in grams) ratio there was evidence of an interaction between initial core-colonization and burn soil treatment; therefore differences in the ratios between core-colonized and core-uncolonized seedlings were estimated within each soil treatment. For seedlings in sterilized burn soil, the foliar C:N ratio of core-colonized seedlings on average was 6.34 or 24% greater than that of core-uncolonized seedlings ($p = <0.001$), or a ratio of 33:1 compared to a ratio of 27:1. In unsterilized burn soil, the foliar C:N ratio of core-colonized seedlings on average was 3.36 or 11% greater than that of core-uncolonized seedlings ($p = 0.028$), or a ratio of 33:1 compared to a ratio of 29:1. Greater foliar C:N ratios are a result of proportionally higher amounts of carbon in core-colonized seedlings. Averages and standard deviations are presented in Table 2.6; p-values are presented in Table 2.7.

Overall, core-colonized seedlings had higher foliar nitrogen content, higher foliar carbon content, and increased foliar C:N ratios. Core-colonized seedlings were depleted

in foliar $\delta^{15}N$ and enriched in foliar $\delta^{13}C$ in comparison to core-uncolonized seedlings (Table 2.7).

Table 2.6. Results of foliar nutrient and isotope analysis with means and standard deviations by core-colonization status and burn soil treatment.

	Colonization Status	%N	%C	N content (g)	C content (g)	C:N ratio	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)
Sterilized Burn Soil	core-uncolonized	1.86 ± 0.23	49.5 ± 1.6	0.11 ± 0.02	2.94 ± 0.84	27.0 ± 4.0	-1.17 ± 0.55	-31.7 ± 0.7
	core-colonized	1.53 ± 0.19	50.1 ± 2.5	0.14 ± 0.02	4.80 ± 1.11	33.3 ± 5.1	-1.60 ± 0.32	-31.1 ± 0.9
Unsterilized Burn Soil	core-uncolonized	1.71 ± 0.20	49.7 ± 1.2	0.12 ± 0.03	3.67 ± 1.21	29.4 ± 3.5	-0.92 ± 0.57	-31.6 ± 0.5
	core-colonized	1.57 ± 0.17	51.0 ± 2.6	0.15 ± 0.03	4.76 ± 1.02	32.7 ± 3.4	-1.29 ± 0.54	-30.5 ± 0.5

Table 2.7. Estimated differences, 95% confidence intervals, t-values, and significance for seedling parameters compared across core-colonized and core-uncolonized seedlings. Difference is positive if the parameter is larger for core-colonized seedlings.

Seedling Parameter	Treatment	Estimated Differences, 95% CI (core-colonized vs core-uncolonized)	T-stat	Significance
Foliar N Concentration (%)†	sterilized burn soil	-0.33 (-0.46, -0.21)	-5.31	**<0.001****
	unsterilized burn soil	-0.14 (-0.28, -0.004)	-2.05	**0.044***
Foliar N Content (g)††	averaged across sterilized and unsterilized burn soil	0.03 (0.018, 0.041)	4.88	**<0.001****
Foliar $\delta^{15}N$ (‰)††	averaged across sterilized and unsterilized burn soil	-0.41 (-0.63, -0.19)	-3.66	**<0.001****
Foliar C Concentration (%)†	averaged across sterilized and unsterilized burn soil	0.88 (-0.18, 1.94)	1.65	0.104
Foliar C Content (g)†††	sterilized burn soil	1.86 (1.18, 2.54)	5.33	**<0.001****
	unsterilized burn soil	1.09 (0.34, 1.84)	2.84	**0.0052***
Foliar $\delta^{13}C$ (‰)†††	sterilized burn soil	0.66 (0.21, 1.12)	2.88	**0.0046***
	unsterilized burn soil	1.14 (0.64, 1.64)	4.49	**<0.001****
Foliar C:N Ratio†	sterilized burn soil	6.34 (3.62, 9.06)	4.66	**<0.001****
	unsterilized burn soil	3.36 (0.38, 6.35)	2.25	**0.028***

Notes: Significant at * $P < 0.05$ and ** $P < 0.001$

† For foliar N and C concentration and C:N ratio, comparisons are made within each burn soil treatment (sterilized and unsterilized) if there is significant evidence of a 2-way interaction between soil treatment and core-colonization status ($p < 0.05$), and are averaged if there is no evidence.

†† Due to a 2-way interaction in the analysis of foliar N content and $\delta^{15}N$, estimates were made for each response averaged across soil treatments.

††† Due to a 3-way interaction in the analysis of foliar C content and $\delta^{13}C$, estimates were made for each response within each soil treatment between core-colonized and core-uncolonized seedlings.

Relation of Foliar Nutrient and Isotope
Status to Number of Ectomycorrhizae

The actual number of ectomycorrhizae on new root growth of seedlings was related to foliar nitrogen content (g) and $\delta^{15}N$ (‰) as well as foliar carbon content (g) and $\delta^{13}C$ (‰) of core-uncolonized and core-colonized seedlings in sterilized and unsterilized burn soils (Figure 2.13).

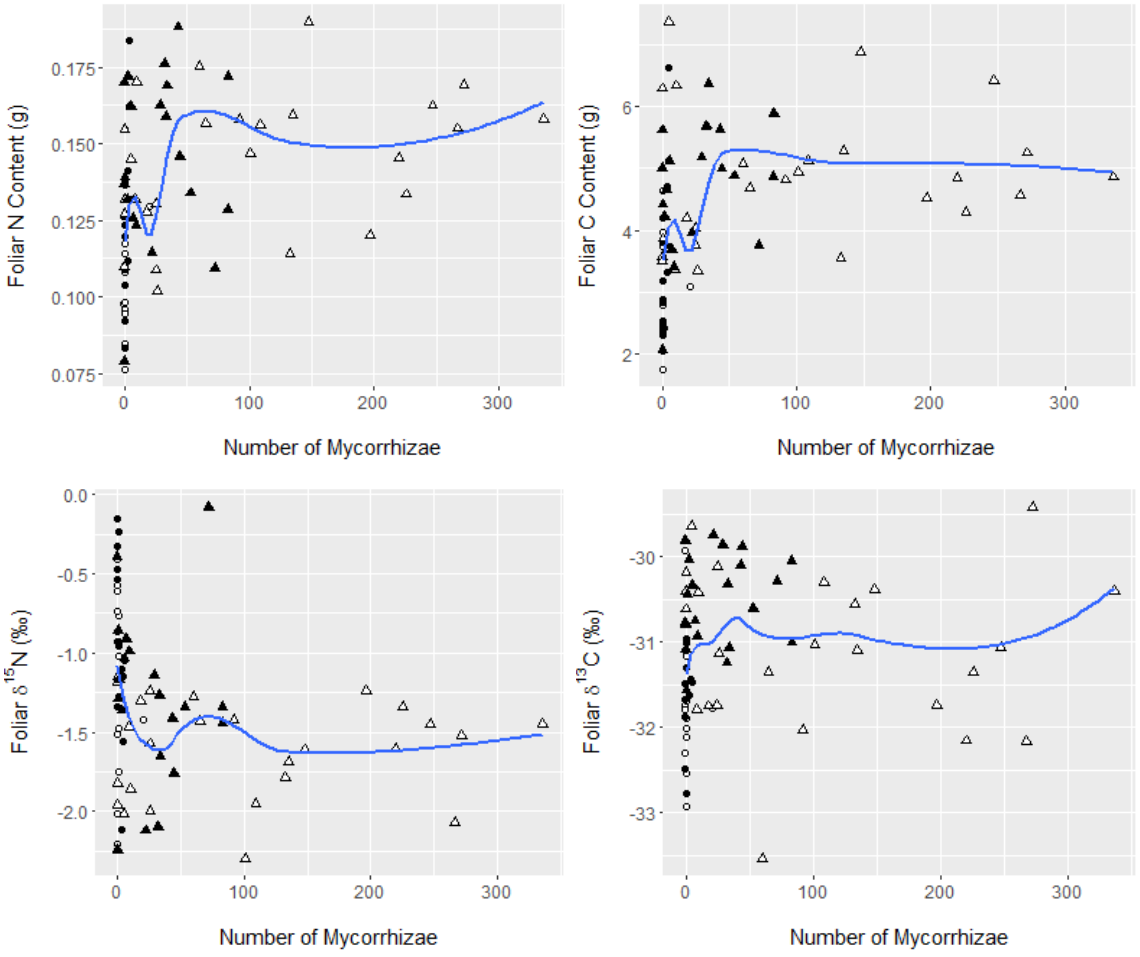

Figure 2.13: Scatterplots of foliar nitrogen content and $\delta^{15}N$ (left column) and foliar carbon content and $\delta^{13}C$ (right column) by number of ectomycorrhizae colonizing new root growth of seedlings for treatment combinations: sterilized burn soil, core-colonized (Δ), unsterilized burn soil, core-colonized (▲), sterilized burn soil, core-uncolonized (○), unsterilized burn soil, core-uncolonized (●).

Nitrogen content initially rises with greater numbers of mycorrhizae and then levels off above 50 ectomycorrhizae. As exhibited by trend lines, the opposite is true for $\delta^{15}N$ which initially decreases as the number of mycorrhizae increases and then levels off. Carbon content initially rises with greater numbers of mycorrhizae while there is a simultaneous enrichment in $\delta^{13}C$, as exhibited by parallel trend lines; both level off above 50 ectomycorrhizae. In general, core-colonized seedlings formed more mycorrhizae on new root growth in sterilized burn soil than in unsterilized burn soil. Core-colonized seedlings tended to have a higher foliar nitrogen and carbon content and were enriched in $\delta^{13}C$ and depleted in $\delta^{15}N$ in comparison to core-uncolonized seedlings. Core-uncolonized seedlings tended to have a lower foliar nitrogen and carbon content and were enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$ in both burn soil treatments.

Discussion

The use of *Suillus* fungi in the restoration of whitebark pine through the outplanting of inoculated seedlings on burn sites has only recently begun to be explored (Lonergan et al. 2014, Asebrook and Hintz 2015). Destructive sampling of seedlings as a follow-up to outplanting, in order to measure seedling parameters and actual ECM colonization, is often not possible. In this study, a controlled greenhouse setting was utilized to examine the effects of root colonization by *Suillus* fungi on whitebark pine seedlings planted in natural burn soil. Although it was necessary to dilute the burn soil due to drainage concerns and seedlings were well-watered, results from this experiment still have implications for applied field plantings. At the start of this experiment, it was

not known if the *Suillus* species typically used in field restoration could actually colonize seedling roots in burn soil in a timely manner. The study showed that *Suillus* was capable of colonizing new roots in all soil treatments, at least under greenhouse conditions. This suggests that *Suillus* fungi colonizing the core root systems of nursery grown whitebark pine seedlings have the potential to grow out into burn soil along with new roots once the seedlings are planted. Additionally, colonization of seedling roots with *Suillus* species significantly impacted most seedling parameters.

Ectomycorrhizal Colonization of Seedlings

Upon arrival to the Montana State University PGC, approximately half of the whitebark pine seedlings for this experiment were already well colonized by ECM fungi. Molecular identification of these fungi revealed two species, *Suillus sibiricus* and *Suillus discolor*, both of which are known associates of whitebark pine (Mohatt et al. 2008). It is not uncommon to find ectomycorrhizal fungi colonizing nursery pine seedlings but the quantity and diversity of fungi depends on the host species and the ability of fungi to thrive under nursery conditions (Quoreshi et al. 2009). Common nursery fungi that have been found in association with other pine seedlings include ectendomycorrhizal "E-strain" (*Wilcoxina*) species (Mikola 1988), the ectomycorrhizal fungus *Thelephora terrestris* and certain suilloid species, in particular those of *Rhizopogon* (El Karkouri et al. 2005, Menkis et al. 2005, Iwański et al. 2006). Whitebark pine seedlings grown at the Coeur d'Alene Nursery occasionally host strong populations of suilloid fungi, which are not considered typical nursery fungi and their origin is considered to be local from surrounding 5-needle pines (Cripps and Jenkins 2015). *Thelephora* is also possible at the

nursery, but was not on seedlings used in the experiment as assessed by morphological (absence of clamps and cystidia) and molecular methods; E-strain was minimal.

After seedlings were grown for 6 months in experimental burn soil treatments, the final mycorrhizal assessment of new root growth surprisingly did not detect any additional fungi in the unsterilized burn soil. The vast majority (99.9%) of ectomycorrhizae observed growing on the new roots of seedlings in burn soil were of the *Suillus* morphotype which matched that of the original colonizers of the seedling core root systems. While *Amphinema* and "E-strain" fungi have been found to associate with natural and planted whitebark pine seedlings on a burn (Trusty and Cripps 2011), and so were expected, only a few possible E-strain mycorrhizae were observed. *Cenococcum*, a generalist ECM fungus that associates with naturally regenerating whitebark pine seedlings (Hasselquist et al. 2005, Mohatt et al. 2008, Trusty and Cripps 2011), also was not observed on any of the seedlings. One possible explanation for an absence of detected ECM fungal diversity in the burn soil is that the *Suillus* species already colonizing the core root systems of the seedlings out-competed any fungi in the burn soil on new roots; however, core-uncolonized seedlings planted in unsterilized burn soil also did not pick up any additional fungi. This suggests that the burn was severe enough to kill any ectomycorrhizal propagules in the soil, and also the soil was only collected to a depth of 20 cm from a burn that was classified as high severity (BDNF 2013). ECM fungal populations are generally reduced after wildfire but this is dependent upon burn severity (reviewed in Cairney and Bastias 2007). One study examining ECM fungal diversity after disturbances in Scots pine stands in Sweden found that after hard burns, almost no

mycorrhizas were found on root material in soil samples taken at a 15 cm depth (Dahlberg et al. 2001). However, Glassman et al. (2015) showed that ECM spore banks, while reduced in richness, can survive severe fires. Alternatively, in the current study, any fungi in the soil might not have responded to nursery conditions.

Over the course of the experiment 10 seedling pairs in containers that were originally categorized as core-uncolonized exhibited colonization on new root growth, and this includes those in sterilized soil. The morphology of the newly developing ectomycorrhizae were identical to the original *Suillus* morphotype on initially colonized seedlings, and they likely originated from this source; thus these few seedlings originally classified as uncolonized, likely were colonized originally. It is possible that there were undetected *Suillus* species in the unsterilized soil that could not be distinguished from original colonizers, but we consider this unlikely. These 10 seedlings were removed from statistical analyses in order to better test the hypothesis.

Additionally, 25 of the 84 seedling pairs in containers that were initially categorized as core-colonized did not exhibit colonization on new root growth; 15 of these 25 seedling pairs (60%) had some ectomycorrhizal development, but because the root systems were <1% colonized they were not categorized as colonized. Thus it appears that this colonization was progressing slowly. Because these 25 seedling pairs were initially significantly colonized, they were included in the analyses as colonized seedlings.

In this study the ‘treatment’ of ectomycorrhizal colonization of seedlings was observationally recorded as ‘colonized’ or ‘uncolonized’, and seedlings were not

inoculated except in soil treatment 4. Though high frequencies of colonization have been achieved in the inoculation of whitebark pine seedlings with *Suillus* species, it is important to note that inoculation does not always result in colonization of roots (Cripps and Grimme 2011). Other studies have similarly shown variation in the success of inoculation due to several factors such as host suitability, growing substrate, and inoculation rate (Davey et al. 1990, Parladé et al. 2004, Brundrett et al. 2005, Rincón and Fernández-Pascual 2007). Due to constraints, it has not been possible to assess actual root colonization after inoculation prior to outplanting in field studies using whitebark pine seedlings (Lonergan et al. 2014, Asebrook and Hintz 2015). As a result, the effect of actual ECM colonization on outplanted seedlings may be obscured.

Soil Planting Treatments

Four soil treatments were originally used in this experiment to optimize the possibility that ectomycorrhizal colonization would take place in at least some of the soils. Substantial ectomycorrhizal colonization was observed on new roots in all soil treatments (including burn soil and soil mix 3), but some differences were observed.

For core-colonized seedlings planted in unsterilized soil (soil treatment 2), frequency (40%) and abundance (12%) of new root colonization were lower than for core-colonized seedlings planted in sterilized soil (soil treatment 2, 73% and 28% respectively), indicating that the formation of *Suillus* ectomycorrhizae may have been repressed in the unsterilized burn soil. This could be an artifact of sampling bias or a result of an induced biotic repression in unsterilized soil. Studies have shown that in some cases, mycelia grow faster in sterilized soil (Skinner and Bowen 1974) and it is well

known that soil bacteria can either inhibit or encourage ECM fungal growth and colonization (Bowen and Theodorou 1979, Fitter and Garbaye 1994, Tarkka and Deveau 2016). Some bacteria are less sensitive to disturbance or can recover more quickly than fungi, so it is likely that the ratio of fungi:bacteria may decrease after a fire (Hammam et al. 2007, Dooley and Treseder 2012). In either case, new root colonization was slower in the unsterilized burn soil of soil treatment 2. In contrast, core-colonized seedlings planted in unsterilized soil after inoculation (soil treatment 4) had comparatively high levels of colonization (frequency 91%, abundance 20%), indicating that colonization was not repressed in this unsterilized burn soil.

For core-colonized seedlings planted in 100% soil mix 3 (soil treatment 3), as a kind of control, frequency (75%) and abundance (15%) of new root colonization were comparable to results from a previous study in which whitebark pine seedlings inoculated with a fresh spore slurry were planted in the same soil mix and supplied a low-N fertilizer (Lonergan and Cripps 2013).

In soil treatment 4, seedlings inoculated with fresh spore slurry (in addition to any original colonization) that were planted in unsterilized burn soil (soil treatment 4) were used to simulate ongoing field studies. The frequency (91%) and abundance (20%) of the new roots of core-colonized seedlings was high, though it is unclear if this is a result of core-colonization or the inoculation, or both. However, only 2 out of 13 core-uncolonized seedlings in this planting treatment exhibited colonization of new root growth, which suggests that inoculation may not have had time to affect the frequency of new root colonization. Therefore, results suggest it may be essential to establish ectomycorrhizal

colonization in a nursery setting several months prior to outplanting rather than attempting inoculation immediately before planting; this initial colonization appears important for subsequent efficient colonization of new root growth in the field.

Seedling Parameters

Overall, seedlings colonized by *Suillus* fungi were taller and had larger stem diameters and biomass, which is rather stunning given that this is a slow-growing pine species. A soil effect was not expected but was observed for most parameters so it was included as an interaction term in the analyses. Differences were observed in seedling parameters between colonized and uncolonized seedlings in both soil types, but they were almost always more substantial in the sterilized soil. This could be a result of the overall higher average abundance (as well as the higher actual number of ECM) of new root colonization on seedlings planted in sterilized soil, that was reflected in more significant changes in seedling parameters. This is likely a function of the slower mycelial growth and thus slower colonization in unsterilized soil (Skinner and Bowen 1974, Bowen and Theodorou 1979, Fitter and Garbaye 1994). Although not as well colonized in the time period, seedlings planted in unsterilized burn soil are more representative as to what may occur on seedlings planted in the field.

Colonized seedlings were significantly greater in height and stem diameter than uncolonized seedlings in sterilized burn soil but these parameters were not significantly different in unsterilized soil. Height and stem diameter are often used to estimate seedling quality, but these measurements are dependent upon plant species and cannot be used alone as predictors of outplanting performance (Haase 2008). Seedling height can be

indicative of photosynthetic capabilities, growth rates, and ability to outcompete other vegetation, but taller seedlings may be more sensitive to drought and other harsh climate conditions (Haase 2008). Generally, seedling diameter is considered a better indicator of field survival (Davey 1990, Haase 2008) and this may be true for whitebark pine in particular, which is often planted on high-elevation sites in harsh conditions where stem diameter and lateral branching may be more indicative of seedling vigor (Burr et al. 2001). There were no significant differences in the height and stem diameter of seedlings planted in unsterilized burn soil, although colonized seedlings tended on average to be taller. This likely reflects slower colonization in unsterilized soil.

Biomass is another morphological indicator of seedling vigor and potential outplanting performance (Haase 2008). Colonized seedlings were 61% greater in total biomass than uncolonized seedlings in sterilized soil and 27% greater in unsterilized soil. When total biomass was separated into shoot and root biomass, these percentages were similar. Shoot biomass can signify photosynthetic and transpirational capacity and growth potential; root biomass can be an indicator of growth, drought avoidance capability, and survival potential in the field (Haase 2008, Grossnickle 2012).

Shoot:root ratios were not significantly different between colonized and uncolonized seedlings, as colonized seedlings had proportionately greater shoot and root biomass; average shoot:root ratios for all seedlings were approximately 0.7, with roots being almost one and a half times greater in biomass than shoots. Shoot:root ratio is considered an indicator of drought avoidance potential as a balance is needed between shoot transpiration and water uptake from roots (Grossnickle 2012). Ratios ranging from

1.0-3.0 appear to encourage seedling survival with lower ratios signifying greater drought resistance potential, though it is important to consider the quality of the root system as well (Hobbs 1984, Grossnickle 2012). Colpaert et al. (1996) saw an increase in shoot:root ratios in *P. sylvestris* seedlings colonized by ECM (including *Suillus bovinus*) due to suppressed root growth in comparison to shoot growth, though seedlings were very young and were grown semi-hydroponically for a maximum of 12 weeks.

In this experiment, root growth increased with *Suillus* colonization. Though colonized seedlings had greater shoot:new root ratios (or proportionately greater shoot biomass than new root biomass) than uncolonized seedlings, they also had 21% greater new root biomass than uncolonized seedlings. The ability of seedlings to form new root growth has important implications for outplanting. Often, containerized nursery seedling root systems are tightly bound into container shapes which are hydrophobic after being outplanted due to differences between nursery media and natural soil (Bernier et al. 1995, Close et al. 2005). Trusty and Cripps (2011) observed that uninoculated whitebark pine seedlings retained their container-bound root shape and new roots had not grown out into the soil four years after they were planted. Therefore new root growth outside the nursery core bound root system indicates a successful integration into the surrounding soil and can serve as an excellent indicator of drought avoidance and survival potential (Grossnickle 2005, Grossnickle 2012).

Other studies have documented increases in pine seedling parameters due to inoculation with suilloid fungi in a greenhouse setting. Verma et al. (2014) documented an increase in growth and biomass of *Pinus wallichiana* seedlings inoculated with *Suillus*

sibiricus and Hobbie and Colpaert (2003) saw an increase in the biomass of *P. sylvestris* seedlings inoculated with *S. luteus*. Several studies have utilized collected burn soil to conduct spore bank bioassays in the interest of exploring the response of naturally occurring ECM fungi to fire (Baar et al. 1999, Buscardo et al. 2010, Kipfer et al. 2011, Glassman et al. 2015), but fewer have correlated colonization with seedling parameters (Schoenberger and Perry 1982), and all of these have focused on the natural recovery of ECM fungi. Only one study was found in which inoculated and un-inoculated seedlings were grown in a greenhouse in simulation of a restorative outplanting on a burn site (Sousa et al. 2001). In that experiment, *Pinus pinaster* seedlings inoculated with *Suillus*, *Rhizopogon*, and *Pisolithus* that were planted in "burn" soil exhibited greater growth in comparison to un-inoculated seedlings planted in unburned soil; however, the "burn" soil was not natural and wildfire was instead simulated by placing collected soil in a furnace (Sousa et al. 2011). No studies have compared parameters of colonized and uncolonized whitebark pine seedlings planted in collected burn soil in the greenhouse.

Burn Soil

The burn soil mixture in which seedlings were planted in this experiment was estimated to contain 13.6 mg kg^{-1} nitrate. Typically, subalpine conifer forests are nitrogen limited (Körner 2003) and several studies have reported soil NO_3^- levels of less than 1 mg kg^{-1} in conifer stands in the Rocky Mountains (Douglas et al. 2005, Kennedy et al. 2015, Trahan et al. 2015). One study showed that soils collected from whitebark pine stands in the Pioneer Mountains of southwestern MT in the Beaverhead-Deerlodge National Forest also contained less than 1 mg kg^{-1} (Keville et al. 2013). It is well known that soil nitrate

increases due to nitrification of ammonium in the initial years following wildfire, and this level of NO_3^- in the experimental burn soil is consistent with other studies measuring soil nitrate in pine stands post-fire (Covington and Sackett 1992, Choromanska 2000, Knelman et al. 2015). While high nitrogen fertilizer has been shown to inhibit *Suillus* colonization of whitebark pine seedlings (Lonergan and Cripps 2013), this experiment showed that *Suillus* fungi can still colonize whitebark pine seedling roots in burn soil with elevated nitrate levels, which is promising for restoration on burn sites.

Foliar Nutrient Analysis

While seedling morphology should be taken into account when estimating seedling quality, physiological measurements such as nutrient testing can reveal information on the internal functioning of seedlings; when possible to take, these measurements can be useful in predicting outplanting performance (Landis et al. 2005, Haase 2008). In this experiment the concentration of foliar nitrogen was 22% higher in uncolonized seedlings than in colonized seedlings in sterilized soil and 9% higher in unsterilized soil. However, total foliar nitrogen content was 26% higher in colonized seedlings than in uncolonized seedlings when averaged across soil treatments. Measurements of concentration and content are both used in seedling testing, but when seedlings exhibit an enhanced growth rate, concentration measurements can be misleading because mobile nutrients are diluted in growing tissues (Landis et al. 2005, Jones et al. 2009). Therefore the lower concentration of nitrogen in colonized whitebark pine seedlings in this experiment could be a result of faster growth rates (indicated by higher biomass); total nitrogen content may be more indicative of seedling nutrient status.

Kohzu et al. (2000) also found that when comparing foliar nitrogen, *Pinus densiflora* seedlings inoculated with *Suillus granulatus* had lower N concentrations but a greater N content than uncolonized seedlings likely due to a growth dilution effect. Nitrogen content also has a greater predictive value in outplanting performance than concentration (Larsen et al. 1988, Landis et al. 2005). The nutrient content of a seedling can be especially important during the initial time period after outplanting. This is when internal foliar nutrient cycling supplies a critical portion of the nutrients needed for new growth (Millard and Proe 1993, Folk and Grossnickle 2000, Millard and Grelet 2010).

Carbon assimilation and water use efficiency (WUE, estimated by measurements of photosynthesis and transpiration) are related to seedling establishment and growth in treeline whitebark pine communities (Bansal et al. 2011). In the current experiment, foliar carbon concentrations did not differ significantly between colonized and uncolonized whitebark pine seedlings, though colonized seedlings did have slightly higher concentration levels. However, foliar carbon content was 63% higher in colonized seedlings than in uncolonized seedlings in sterilized soil and 30% higher in unsterilized soil. The external source of carbon for plants comes in the form of CO_2 assimilated in foliage in photosynthesis (Millard and Grelet 2010), therefore it makes sense that seedlings with a greater shoot biomass (those that were colonized), would contain more total carbon. However, a more detailed look at carbon assimilation and water relations would be useful in exploring how ECM affect carbon balance (e.g. measuring photosynthetic rates, transpiration, and nonstructural carbohydrates) (Bansal et al. 2011, Reinhardt et al. 2015).

Studies have reported that colonization by some ectomycorrhizal fungi can create an initial carbon sink in seedlings, though this sink is effectively balanced by enhanced photosynthetic rates (Dosskey et al. 1990, Hobbie and Wallander 2006, Nehls et al. 2010). Hobbie and Colpaert (2004) found that *P. sylvestris* seedlings colonized by *S. luteus* under both low and high nitrogen availability contained less total system C (carbon in the shoots, root, mycelium, and perlite) and lower carbon concentration in foliage than non-mycorrhizal seedlings; researchers hypothesized that the *Suillus* colonized plants were experiencing a reduction in metabolic efficiency because greater amounts of C were being allocated to respiration rather than to accumulation. However, in that study seedlings were very young and were grown semi-hydroponically for a maximum of 70 days (Hobbie and Colpaert 2004). This effect was not observed in the current study utilizing whitebark pine seedlings, at least in the analysis of total foliar C, where colonized seedlings had greater carbon content than uncolonized seedlings. In a comprehensive analysis of literature on the physiological costs of mycorrhizal relationships, Corrêa et al. (2012) provide evidence that C is in excess in these relationships and that instead, nutrient acquisition is the main limiting factor, so that mycorrhizal relationships are in fact, not costly to the host plant.

Foliar ratios of C:N were 24% greater in colonized seedlings than in uncolonized seedlings in sterilized soil and 11% greater in unsterilized soil. Generally, colonized seedlings had a C:N ratio of 33:1 and uncolonized seedlings had a ratio of approximately 28:1. This is a result of proportionately more foliar carbon than nitrogen in the colonized seedlings, though colonized seedlings still contained more foliar nitrogen than

uncolonized seedlings. While C:N ratios can be used to estimate the relative growth rates of plants (Peng et al. 2011), it is unclear whether the difference in ratios observed in this study would reflect differences in the relative growth rates. McGroddy et al. (2004) found that on average, coniferous forests had foliar C:N ratios of approximately 60:1 which was significantly higher than tropical and temperate broadleaf forests (35:1); however this study was conducted on fully mature natural conifers. This relatively high C:N ratio for conifers is generally attributed to the N-efficient growing strategy of pines in N-limited habitats and the greater amount of carbon rich secondary compounds such as tannins and resins that conifers produce (McGroddy et al. 2004).

Foliar Nitrogen Isotope Analysis

Stable isotopes of nitrogen and carbon are utilized in various ways to explore the plant-fungal relationship. In biochemical processes occurring in organisms, heavier isotopes such as ^{15}N and ^{13}C are often discriminated against in favor of ^{14}N and ^{12}C (Dawson et al. 2002). Isotopic fractionation, the process in which a substrate may change in its composure of heavy and light isotopes, can reveal information about physiological processes and movements of compounds. Isotopic signatures of $\delta^{15}N$ and $\delta^{13}C$ have been measured in: the sporocarps of macro-fungi to determine ecological function (Hobbie et al. 2012), in the foliage of natural mature trees to explore plant to plant movement of compounds through a mycorrhizal network (Dawson et al. 2002), and more rarely in the foliage of seedlings in controlled settings to correlate mycorrhizal status with plant functioning (Hobbie and Colpaert 2003, Schweiger 2016).

In this study, whitebark pine seedlings colonized by *Suillus* fungi were significantly depleted in foliar $\delta^{15}N$ (0.4‰) in comparison to uncolonized seedlings. It is recognized that in a mycorrhizal relationship, ectomycorrhizal fungi preferentially retain the heavier isotope of nitrogen, $\delta^{15}N$, while passing fractionated nitrogen ($\delta^{14}N$) to the host plant; therefore under controlled settings, the foliar isotopic signature of plants can reflect mycorrhizal status (Hobbie and Högberg 2012). Studies have shown that pine seedlings colonized by *Suillus* fungi can be depleted in foliar $\delta^{15}N$ and that this is dependent upon the availability and form of nitrogen. One greenhouse study found that *Pinus haplensis* seedlings colonized by *Suillus luteus* were depleted in foliar $\delta^{15}N$ by 2.4‰ under a low supply of N (NH_4^+ and NO_3^-) and 0.7‰ depleted under a high supply of N in comparison to uncolonized seedlings (Hobbie and Colpaert 2003). Another study cited significant depletions in foliar $\delta^{15}N$ of *P. densiflora* seedlings inoculated with *S. granulatus* in comparison to non-inoculated seedlings, when grown in a greenhouse setting under the application of a low N (ammonium salt) fertilizer (Kohzu et al. 2000). Hobbie et al. (2008) reports that *P. sylvestris* seedlings inoculated with *S. bovinus* were 3.5‰ depleted in foliar $\delta^{15}N$ when under low rates of NH_4^+ application, 1.7‰ depleted under high NH_4^+, 0.5‰ depleted under low NO_3^-, and 1.3‰ depleted under high NO_3^-. Therefore it appears that fractionation is greatest in the foliage of colonized seedlings under low N availability, with NH_4^+ as the main source of N. *Suillus* colonization of seedlings was greater under a supply of ammonium in comparison to nitrate, hence the greater depletion in foliar $\delta^{15}N$ (Hobbie et al. 2008).

Several studies have shown that plant $\delta^{15}N$ is positively correlated with soil nitrogen availability (Garten and Van Miegroet 1994, Evans 2001, Hobbie and Colpaert 2003, McLauchlan et al. 2007, Craine et al. 2009). High N availability results in less of a reliance on mycorrhizal fungi by the host plant, thus resulting in decreased sequestration of ^{15}N by the fungi (less fractionation); therefore the plant will have increased $\delta^{15}N$ (Hobbie and Högberg 2012). As such, the effects of mycorrhizal colonization on foliar $\delta^{15}N$ should be more obvious where nitrogen is limiting.

Other studies have shown ECM fungi generally prefer ammonium as a nitrogen source, and specifically that isolates of *Suillus* species, including *S. plorans* and *S. sibiricus*, often prefer ammonium over nitrate (excluding a few isolates of *S. placidus* which preferred nitrate) (Keller 1996, Antibus et al. in ed.). Though a majority of *Suillus* fungi are able to metabolize nitrate, it is thought that the energetic cost of reducing nitrate depresses fungal growth; consequently, faster growing isolates of *Suillus* species appear to be those that are better at utilizing a range of nitrogen sources (Nygren et al. 2008, Antibus et al. in ed.). Additionally, *Suillus* fungi have demonstrated a propensity for utilizing amino acids as a form of N which could relate to the habitat and soil type with which these fungi are often associated (Antibus et al. in ed.). However, this is beyond the scope of the current study as only nitrate was measured in the experimental burn soil.

This is the first study to explore the foliar isotopic signature of nitrogen in seedlings planted in natural burn soil in a controlled setting. In this experiment, seedlings colonized by *Suillus* fungi were 0.4‰ more depleted in foliar $\delta^{15}N$ in comparison to uncolonized seedlings when averaged across soil treatments. This amount of $\delta^{15}N$

depletion is less than (but still significant) in comparison to that reported in other greenhouse studies citing fractionation by *Suillus* species not in burn soil (Kohzu et al. 2000, Hobbie and Colpaert 2003, Hobbie et al. 2008). This could be a result of the relatively high nitrate content of the experimental burn soil as increased N availability can result in less of a dependence on ECM fungi, and therefore less sequestration of ^{15}N by the fungi. In the current experiment average foliar $\delta^{15}N$ values for whitebark pine seedlings ranged from -0.92‰ to -1.6‰, depending upon treatment; this is somewhat comparable to foliar $\delta^{15}N$ values for *Suillus* colonized *P. sylvestris* seedlings in other studies where values have ranged from an average of -1.6‰ to -4.2‰ (Hobbie et al. 2008) and -0.84‰ to 1.62‰ (Hobbie and Colpaert 2003), depending upon treatment. However, it is difficult to make direct comparisons across these studies as source $\delta^{15}N$ values were not measured in this experiment.

Total foliar nitrogen content has been shown to negatively correlate with foliar $\delta^{15}N$ in colonized seedlings (Kohzu et al. 2000), as was observed in this experiment (Figure 2.10, r = -0.48). Colonized seedlings had a higher foliar N content and comparably depleted foliar $\delta^{15}N$ values in contrast to uncolonized seedlings which had a lower foliar N content and comparably enriched foliar $\delta^{15}N$ values. This indicates that the whitebark pine seedlings colonized by *Suillus* fungi were receiving nitrogen that was depleted in $\delta^{15}N$ from the mycorrhizal fungi. The actual number of ectomycorrhizae on seedlings was generally positively related to foliar nitrogen content and negatively related to foliar $\delta^{15}N$ up to a threshold of approximately 50 ectomycorrhizae (Figure 2.13).

Beyond this threshold, additional colonization did not result in higher N content or lower $\delta^{15}N$.

Foliar Carbon Isotope Analysis

In this experiment, seedlings colonized by *Suillus* were significantly enriched in foliar $\delta^{13}C$ when planted in both sterilized and unsterilized burn soil. Foliar carbon isotope measurements have been related to varying plant physiological traits but are most often associated with WUE (Farquhar et al. 1989). However, complicating factors can affect WUE and measurements of foliar $\delta^{13}C$ may be more indicative of general photosynthetic capacity and plant functioning (Dawson et al. 2002, Hobbie and Colpaert 2004). During gas exchange, plants can discriminate against $^{13}CO_2$ in the assimilation of carbon, though this is dependent upon environmental conditions, plant physiology and morphology, and genetics; therefore it is difficult to directly correlate single plant traits with $\delta^{13}C$ (Dawson et al. 2002). Another common use of carbon isotopes involves labeling ^{13}C to track C allocation within plants as well as between plants and mutualistic ectomycorrhizae (Dawson et al. 2002)

Although carbon is assimilated by the plant in a mycorrhizal relationship, as opposed to nitrogen which is mostly taken up by the fungus, foliar $\delta^{13}C$ values can still provide information on the plant-fungal relationship. Higher levels of foliar $\delta^{13}C$ have been associated with ectomycorrhizal colonization and nutrient status of plants. Hobbie and Colpaert (2004) suggested that nitrogen availability (instead of water) limits plant growth and the assimilation of C, and that consequently, ectomycorrhizal relationships can indirectly affect the assimilation of C and therefore foliar $\delta^{13}C$. In that study, *P.*

sylvestris seedlings colonized by *S. luteus* at low N availability (in a well-watered system) were approximately 1‰ enriched in foliar $\delta^{13}C$ when compared to non-mycorrhizal seedlings or seedlings colonized by *Thelephora terrestris*; researchers hypothesized that this was likely do to the drawdown of carbon by the fungi, effectively lowering carbohydrate concentrations in the leaves and thereby increasing photosynthetic rates. At high N availability, seedlings were approximately 1‰ enriched in $\delta^{13}C$ in comparison to low N treatments, and no significant differences were observed between mycorrhizal and non-mycorrhizal seedlings. Foliar $\delta^{13}C$ was also positively correlated with foliar nitrogen concentration (%N); this relationship was attributed to higher carbon fixation rates and lower internal CO_2 concentrations due to higher levels of nitrogen in photosynthetic tissues (Hobbie and Colpaert 2004).

In the current experiment, seedlings colonized by *Suillus* fungi were 0.66‰ enriched in foliar $\delta^{13}C$ when planted in sterilized soil and 1.14‰ enriched when planted in unsterilized soil in comparison to uncolonized seedlings. In this experiment average foliar $\delta^{13}C$ values for whitebark pine seedlings ranged from -30.5‰ to -31.7‰ depending upon treatment; this is somewhat comparable to foliar $\delta^{13}C$ values of *Suillus* colonized *P. sylvestris* seedlings in Hobbie and Colpaert's (2004) study which ranged from an average of -27.6‰ to -29.1‰, depending on treatment. However, direct comparisons across these studies are difficult as source $\delta^{13}C$ values were not obtained in this experiment. Though differences were observed in foliar $\delta^{13}C$ between colonized and uncolonized seedlings in the current study, on average needles only accounted for approximately 25% of total seedling biomass (Table 2.4). Roots are generally more enriched in $\delta^{13}C$ in comparison to

needles, likely because mobile transfer compounds are enriched in ^{13}C in comparison to immobile compounds (Brugnoli and Farquhar 2000, Hobbie and Colpaert 2004). Therefore, differences in $\delta^{13}C$ would be more obvious in roots.

A positive correlation was observed between total foliar carbon content and foliar $\delta^{13}C$ in this experiment (Figure 2.12, $r = 0.31$); colonized seedlings had a higher foliar C content and were comparably enriched in foliar $\delta^{13}C$ while uncolonized seedlings had less foliar C content and were comparably depleted in foliar $\delta^{13}C$. In summary, whitebark pine seedlings colonized by *Suillus* fungi contained more total foliar carbon which was likely due to a greater shoot biomass and therefore a greater photosynthetic surface area and incorporation of structural carbons. Colonized seedlings also contained greater amounts of foliar $\delta^{13}C$, possibly due to enhanced photosynthetic rates driven by carbon allocation to the fungi. The actual number of ectomycorrhizae was positively related to foliar carbon content and foliar $\delta^{13}C$ to a threshold of approximately 50 ectomycorrhizae (Figure 2.13) which indicates that beyond a certain threshold, more colonization does not necessarily translate into a greater effect on seedlings; the dip in $\delta^{13}C$ observed in seedlings with approximately 20-30 ectomycorrhizae could be the result of an initial carbon sink during the early stages of colonization.

Summary and Conclusions

In the current widespread effort to restore whitebark pine, planting guidelines recommend that blister rust resistant seedlings should be planted with microsites on recently burned forests that have historically hosted whitebark pine populations (Keane et al. 2012). While much time and effort has been spent in developing an effective

restoration program, the survival of seedlings after outplanting is often low (Izlar 2007). Currently, it is not common practice to establish colonization by native mutualistic ectomycorrhizal fungi on whitebark pine seedlings prior to outplanting. However, it is known that the majority of woody plants rely on ectomycorrhizal associations for the uptake of nutrients (Smith and Read 2008) and ECM fungi are commonly used in the restoration of tree species around the world (Moser 1956, Marx and Cordell 1988, Amaranthus 2002, Schmid 2006, Vosátka et al. 2008, Rainer et al. 2015). It is important to consider all components of a natural system in the effort to restore a threatened species.

Ectomycorrhizal fungal relationships can be particularly important to plant establishment and survival in subalpine nutrient-limited ecosystems such as where whitebark pine occurs (Lilleskov and Bruns 2001, Hawkins et al. 2015). Field surveys of ECM fungal diversity associated with whitebark pine and inoculation trials of seedlings have revealed that *Suillus* species are of high importance (Mohatt et al. 2008, Cripps and Grimme 2011). *Suillus* fungi are especially involved in the assimilation of nitrogen and they can utilize a variety of N forms (Keller 1996, Antibus et al. in ed.). Nutrient acquisition is a primary limiting factor for seedling establishment after outplanting, especially after a disturbance such as wildfire (Hawkins et al. 2015). Initial field studies have shown that inoculation with native *Suillus* fungi prior to outplanting on a burn site can improve whitebark pine seedling survival and possibly increase growth (Lonergan et al. 2014, Asebrook and Hintz 2015). However, destructive sampling is often not possible in the field, and the causes of seedling mortality and success are unclear. This study is the

first to attempt to understand how colonization by *Suillus* fungi affects the physiological processes in whitebark pine seedlings after they are outplanted on a burn by planting seedlings in burn soil in a controlled greenhouse setting.

The effects of colonization by *Suillus* fungi on whitebark pine seedlings planted in natural burn soil and grown for 6 months in a greenhouse setting are summarized here with implications for restoration.

In contrast to uncolonized seedlings:

- Colonized seedlings exhibited increased height, stem diameter, and biomass which indicates improved growth that might benefit establishment. Differences were greater in sterilized soil, possibly due to faster/greater amounts of suilloid colonization.
- Colonized seedlings had more new root growth in burn soil which could benefit seedling establishment and drought stress tolerance after outplanting.
- Foliar nitrogen concentrations were lower in colonized seedlings, likely due to a dilution effect as a result of greater growth/biomass in colonized seedlings. Foliar nitrogen content was higher in colonized seedlings, which suggests possible improved outplanting performance.
- There was no difference in foliar carbon concentrations between colonized and uncolonized seedlings. Foliar carbon content was higher in colonized seedlings, likely due to greater shoot biomass.

- Colonized seedlings were more depleted in foliar $\delta^{15}N$ which was attributed to fractionation of ^{15}N by the *Suillus* fungi on roots.
- Colonized seedlings were enriched in foliar $\delta^{13}C$ which is attributed to enhanced photosynthesis driven by the carbon sink created by the *Suillus* fungi on roots.
- Foliar nutrient contents were correlated to foliar isotope measurements. N content was negatively correlated with $\delta^{15}N$ and C content was positively correlated with $\delta^{13}C$.
- The actual number of ectomycorrhizae related to foliar nutrient and isotope measurements to a threshold of approximately 50 fungi, indicating that after initial colonization a greater abundance of ECM does not necessarily linearly affect seedling parameters.

Overall, whitebark pine seedlings colonized by *Suillus* fungi exhibited increased growth and elevated nutrient status. Isotopic analysis indicated that differences were actually due to ECM colonization. Though this was a controlled greenhouse experiment and burn soil was diluted with a greenhouse soil mixture, establishing colonization of whitebark pine seedlings by *Suillus* fungi prior to outplanting on a burn site may benefit seedlings during the initial establishment period. The ectomycorrhizal assessment of new root growth indicated that *Suillus* fungi have the ability to grow into and persist on seedling root systems at least for a short period of time in burn soil, and the incorporation of new root growth into the surrounding natural soil is a very important component of seedling establishment (Grossnickle 2012).

If these effects of ectomycorrhizal colonization in a greenhouse setting are sustainable in a field planting, this study has important implications for the restoration of whitebark pine. Establishing mycorrhizal colonization on whitebark pine seedlings by native ectomycorrhizal *Suillus* fungi prior to outplanting has the potential to increase initial growth and establishment of seedlings on burn sites, which may enhance long term survival. Even small increases in seedling survival can have great impacts on the efficiency of restoration and optimization of seedling resources in the effort to maintain whitebark pine across its natural range.

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CHAPTER THREE

FIELD STUDY: NATIVE ECTOMYCORRHIZAL FUNGI AND SPATIAL ANALYSES FOR THE RESTORATION OF WHITEBARK PINE ON THE EUREKA BASIN BURN

Introduction

Whitebark Pine

Whitebark pine (*Pinus albicaulis*) is a five-needle stone pine endemic to western North America where it functions as a keystone species in subalpine forest ecosystems (Arno and Hoff 1989). A large portion of the range of whitebark pine lies in the northern Rocky Mountains where it covers approximately 10-15% of the forested landscape (Arno 1986, Tomback et al. 2001). This long-lived slow growing tree is an iconic species that provides numerous ecosystem services and is also appreciated for its aesthetic quality. As a five-needle stone pine, it bears indehiscent cones which contain nutrient-rich pine nuts (wingless seeds) that are a valuable food source for a variety of animals including grizzly and black bears, squirrels, and birds (Hutchins and Lanner 1982, Lanner and Gilbert 1994, Fortin 2011). Whitebark pine is a pioneer on harsh high elevation habitats at treeline where it facilitates the formation of tree island communities (Resler and Tomback 2008, Tomback et al. 2014), helps regulate snowmelt (Farnes 1990), and reduces erosion (Weaver 2001).

In addition to being a dominant species in climax treeline communities (Tomback et al. 2014), whitebark pine occurs in mixed seral stands at lower elevations (Arno and

Hoff 1989, Campbell and Antos 2003). In these lower elevation stands, wildfire functions as a driving force of succession (Arno and Hoff 1989) to which whitebark pine is well adapted because of its unique dispersal ecology. The Clark's nutcracker serves as the main disperser of whitebark pine seeds and completes what Keane et al. (2012) refer to as the "high-mountain ecological triangle": whitebark pine, wildfire, and nutcrackers. The Clark's nutcracker tends to cache seeds in open areas such as burns that have visual cues which aid in seed recovery; this gives whitebark pine an opportunity for rapid recolonization before other wind-dispersed conifers can arrive (McCaughey 1990, Tomback et al. 1993, Lorenz et al. 2008). However, whitebark pine is not limited to a pioneering role as other studies have found that regeneration is similar on paired burned and undisturbed sites (Moody 2006, Larson and Kipfmueller 2010).

In whitebark pine forests, mean fire return intervals can range from 13 to 400+ years depending on fire regimes, stand conditions, weather conditions and topography (Campbell et al. 2011). Fire suppression over the last century is one of many recent threats to whitebark pine populations, especially in lower elevation seral stands which traditionally have shorter fire return intervals (Campbell et al. 2011). Studies have reported that a decrease in the number of subalpine fires that are allowed to burn results in subsequent replacement of whitebark pine by subalpine fir and other competing tree species through extended succession (Murray et al. 2000, Keane 2001). Though whitebark pine can persist in late seral stands (Campbell and Antos 2003), overall fire suppression and longer return intervals appear to lead to a greater reduction of whitebark pine populations.

Other threats to whitebark pine include native mountain pine beetles (MPB, *Dendroctonus ponderosae*), invasive white pine blister rust (WPBR, *Cronartium ribicola*), and climate change (Keane et al. 2012). In recent years, mountain pine beetle populations have reached epidemic levels and reports are of unprecedented whitebark pine mortality (Gibson et al. 2008). In an aerial photo inventory of the Greater Yellowstone Ecosystem (GYE), MacFarlane et al. (2013) estimated that 46% and 36% of the area covered by whitebark pine had experienced severe and moderate mortality respectively. Additionally, modeled climate projections indicate that beetle populations will likely continue to expand under favorable conditions such as warming winters and reduced summer precipitation (Logan et al. 2010, Buotte et al. 2016).

White pine blister rust is an invasive pathogen that has a complicated life cycle of five spore types and two alternating hosts (McDonald and Hoff 2001, Keane et al. 2012). The fungus tends to infect and kill the top cone-producing branches first, thereby directly reducing seed production (McKinney and Tomback 2007). The range of WPBR now nearly encompasses the entire range of whitebark pine and some areas have seen up to a 100% infection of trees (Resler and Tomback 2008, Schwandt et al. 2010, Keane et al. 2012), which is likely to be exacerbated by climate change (Koteen 2002). Though infection rates in the GYE were historically lower than in the northern range of whitebark pine, data suggest that these rates are increasing and it is now estimated that 20-30% of trees in the GYE are infected with rust (Keane et al. 2012, Shanahan et al. 2014). While climate change has the potential to impact whitebark pine indirectly through the spread of MPB and WPBR, climate modeling also suggests there may be a direct decrease in

suitable habitat for whitebark pine in the coming decades (Warwell et al. 2006, Chang et al. 2014).

Restoration of Whitebark Pine

The recent decline of whitebark pine has led to range-wide restoration efforts, mainly through the outplanting of blister rust resistant seedlings (Keane et al. 2012). In the largest whitebark pine seedling monitoring project to date, Izlar (2007) reported that as of 2005, over 210,000 seedlings had been planted in the western United States. Waring and Goodrich (2012) report that from 2006-2011, an additional 1,550 acres had been planted based on personal communications with nursery managers, and this does not include plantings in Canada. Also, the number of plantings has increased since 2011 after whitebark pine was listed as a candidate under the Endangered Species Act (Nicholas and Katzenberger 2011, USFWS 2011), and it is already considered endangered in Canada (Government of Canada 2012).

The production of whitebark pine seedlings for outplanting is a complex process that begins with the identification of phenotypically blister rust resistant parent trees in the field and the protection and subsequent collection of seed from identified trees in the fall (Mahalovich and Dickerson 2004). Progeny are tested for inherited WPBR resistance at the nursery through several rounds of artificial rust inoculation and the most suitable are selected for outplanting (Mahalovich et al. 2006). Nursery practices are constantly evolving with the goal of growing seedlings to the highest standards for outplanting and the establishment of seed orchards (Mahalovich and Dickerson 2004).

Unfortunately, long term survival of whitebark pine seedlings after outplanting has been low. In Izlar's (2007) monitoring study of over 114,000 seedlings, the overall first year survival rate was 74% but this dropped to 38% in years 3-15 after planting. In Glacier National Park, monitoring of plantings found that prior to 2010 average annual survival rates ranged from 31-65% and from 2010-2014, rates range from 25-95% (Asebrook and Hintz 2015). Land managers are intent on improving these survival rates and planting practices are constantly evolving in order to capitalize on seedling resources. Current planting recommendations include planting seedlings on burned areas that have been cleared of competition as this has been shown to increase survival rates (Izlar 2007, Asebrook and Hintz 2015). Additionally, it is recommended that seedlings be planted with microsites such as logs and woody material for protection from harsh weather conditions; where there is adequate moisture; and where whitebark pine populations have declined so drastically that natural regeneration might not occur (McCaughey et al. 2009, Leirfallom 2014).

Although dependent on burn severity, planting on burns is advantageous because of reduced competition, the availability of microsites, and the alteration of soil conditions and nutrient availability (Keane et al. 2012, Bansal et al. 2014). Inorganic nitrogen increases in the soil immediately after wildfires due to the release of ammonium (NH_4^+) by combustion and subsequent nitrification of ammonium into nitrate (NO_3^-) (Covington and Sackett 1992, Certini 2005, Delwiche 2010). Planting soon after a wildfire may ensure that seedlings have access to sufficient inorganic nitrogen; however, if not utilized quickly these inorganic forms of N may become unavailable as the nitrate leaches

downwards and the ammonium is adsorbed by soil (Certini 2005). The duration of time for which these nitrogen effects persist after fire can vary. One study showed that these effects had dissipated by the second growing season in a burned *P. muricata* forest (Grogan et al. 2000); another found that increases in N had been lost by the fifth year following a burn in a *P. ponderosa* stand (Covington and Sackett 1992). Bansal et al. (2014) reported that seedlings of four different tree species including *Pinus sylvestris* exhibited positive ecophysiological responses (growth, photosynthesis, and foliar nutrient concentrations) when they were planted on moderate to severe burns but not when they were planted on those of low severity; this was attributed mainly to a reduction in competition and a the pulse of plant available N. These results could be of particular significance for whitebark pine which typically occurs in high elevation forests in nutrient poor soils (Hansen-Bristow et al. 1990).

Whitebark Pine and Ectomycorrhizal Fungi

Ectomycorrhizal (ECM) fungi are necessary to the life and growth of the majority of woody tree species in nature (Smith and Read 2008). These fungi form structures on root tips of trees called ectomycorrhizae; these structures consist of hyphae that surround individual root cortical cells as well as the whole root tip (Brundrett et al. 1996, Smith and Read 2008). Vast networks of hyphae also grow out into the soil that absorb nutrients, shuttling some to the host tree in exchange for photosynthetically derived carbohydrates (Allen et al. 2003, Smith and Read 2008). In addition to increasing the overall surface area of tree root systems for nutrient uptake, ectomycorrhizal fungi are

able to access a range of nutrient forms otherwise unavailable to the plant (Hobbie and Högberg 2012).

Only one study has attempted to examine the diversity of ECM fungi associated with whitebark pine; this study was restricted to the GYE and it revealed fewer than 50 species of ECM fungal associates (Mohatt et al. 2008). While limited sampling might explain the low diversity of fungi, researchers point out that this number is comparable to that found with other host trees in harsh habitats (Mohatt et al. 2008). For example, one study reported only 10 species of ECM fungi with bristlecone pines (*P. longaeva* Bailey) in California (Bidartondo et al. 2001). In the whitebark pine study, fungi in the suilloid clade, mainly *Suillus* and *Rhizopogon* species, were identified through the presence of both sporocarps and ectomycorrhizae on roots and were found to be of particular significance (Mohatt et al. 2008). These genera are closely related and many of their species are specific to particular tree species in Pinaceae (Bruns et al. 2002). For example, some *Suillus* species are known to associate only with five-needle pines and/or stone pines. Thus far, only *Suillus sibiricus* (Singer) Singer (= *S. americanus* (Peck) Snell), *Suillus subalpinus* M.M. Moser, and *Suillus discolor* (A.H. Sm, Thiers & O.K. Miller) N.H. Nguyen have been confirmed with whitebark pine in the Rocky Mountains (Mohatt et al. 2008, Cripps and Antibus 2011, Nguyen et al. 2016). Of these, *S. sibiricus* is also found with Asian and European stone pines (Moser 2004), while *S. subalpinus* has only been reported with whitebark pine. Other species of ECM with whitebark pine may have a wider host range.

Ectomycorrhizal species that occur simultaneously on the same host are often not functionally redundant and may avoid competition through specialization (Bruns et al. 2002). In the case of *Suillus*, host specialization is evolutionarily derived and likely stems from the differing functional roles of various species (Bruns et al. 2002, Cripps and Antibus 2011). Studies have shown that *Suillus* species in particular are involved in the uptake of nitrogen and that they can utilize a variety of organic forms not directly accessible to the host (Keller 1996, Nygren et al. 2008, Antibus et al. in ed.). This could be of particular importance in subalpine ecosystems where whitebark pine occurs because inorganic nitrogen is often a limiting factor for plant growth (Hansen-Bristow et al. 1990, Körner 2003, Kranabetter 2014, Hawkins et al. 2015, Antibus et al. in ed.).

Ectomycorrhizal Fungi in Restoration

Ectomycorrhizal fungi are commonly used for restoration purposes through the inoculation of seedlings prior to outplanting (Marx and Cordell 1988, Castellano 1996, Amaranthus 2002). *Rhizopogon* species are commonly used to inoculate conifers because of their ability to colonize a wide variety of hosts across a wide range of habitats in comparison to the more restricted *Suillus* species (Amaranthus 2002, Steinfeld et al. 2003, Teste et al. 2004). However, it is important to carefully select suitable native fungi for the restoration of particular tree species that have limited distributions in specific habitat types (Vosátka et al. 2008, Cripps and Grimme 2011).

In Austria, *Suillus* species, including *S. plorans* (Rolland) Kuntze, *S. sibiricus* Singer, and *S. placidus* (Bonord.) Singer, have been used to restore the European Stone pine (*Pinus cembra* L.) in high elevation forests (Moser 2004, Weisleitner 2008).

Inoculation of seedlings prior to outplanting improved survival rates by 20-80% for up to 30 years after planting (Moser 1956, Schmid 2006). Decades later, surveys identified the original fungal inoculants on the roots of the mature trees, indicating that the colonization is sustainable (Rainer et al. 2015). The Austrian Federal Forest Nursery has maintained this procedure for over 50 years (Weisleitner 2008).

Other studies have documented the benefits of using *Suillus* fungi in the restoration of other pines. One study found that the second-year survival rates of *Pinus haplensis* seedlings increased 41-63% when seedlings were inoculated with *Suillus collinitus* prior to outplanting (Rincón and Fernández-Pascual 2007). Franco et al. (2014) found that *P. pinaster* seedlings inoculated with a variety of ECM fungi including *S. bovinus* and *S. granulatus* and outplanted on a burn site showed improved growth in comparison to un-inoculated seedlings. Stenström and Ek (1990) also report that *P. sylvestris* seedlings inoculated with ECM fungi including *S. variegatus* showed improved growth after outplanting.

Though *Suillus* fungi have been effective in these few experimental outplanting studies with other pines, they have not been widely applied and tested in the restoration of whitebark pine. Inoculation trials at Montana State University have identified native *Suillus* species, in particular *S. sibiricus*, as vigorous colonizers of whitebark pine seedlings in the greenhouse (Cripps and Grimme 2011). Though typical fertilizer regimes have been shown to suppress ECM colonization of seedlings (Khasa et al. 2001, Rincón and Fernández-Pascual 2005, Cripps and Grimme 2011), maintenance of *Suillus* colonization of whitebark pine seedlings is possible in the greenhouse with use of a low

nitrogen fertilization regime (Lonergan et al. 2013). Initial monitoring data from a few plantings indicates that inoculation with these *Suillus* fungi prior to outplanting has the potential to improve survival rates. In a study conducted in Waterton Lakes National Park, Lonergan et al. (2014) showed that whitebark pine seedling survival was improved when seedlings were planted with microsites, on prescribed burn areas, and when inoculated with *S. sibiricus* prior to outplanting. Additionally, preliminary results from plantings in Glacier National Park show that whitebark pine seedling survival is improved with *S. sibiricus* inoculation and that growth may also increase (though this is not yet statistically significant) (Asebrook and Hintz 2015).

Inoculation of whitebark pine seedlings prior to outplanting may be especially important when planting on burn sites, as fire can reduce ECM populations (Cripps and Grimme 2011). Ectomycorrhizal fungal diversity decreases after fire, though this is dependent upon the ECM community as well as on fire severity (Smith et al. 2005, Cairney and Bastias 2007). Studies have shown that certain *Rhizopogon* species may be resistant to fire (Baar et al. 1999, Izzo et al. 2006, Peay et al. 2009), but *Suillus* species appear to have less resistance. Bruns et al. (2002) found that the *S. pungens* genets present in a pre-fire *P. muricata* forest were not found after a stand-replacing fire. Glassman et al. (2015) found that the occurrence of *Suillus* was greatly reduced in soil from a severely burned *P. ponderosa* forest in comparison to pre-fire soil. Additionally, Jones et al. (2010) found that the relative abundance of *Suillus* and *Rhizopogon* ectomycorrhizal root tips on naturally regenerating Douglas-fir seedlings was 0% after a low severity fire, though the pre-fire relative abundance of these species was 9%. Trusty

and Cripps (2011) found that suilloid ectomycorrhizae were less frequent (10-13%) on whitebark pine seedlings naturally regenerating on a burn site in comparison to seedlings in an adjacent non-burned area (25%) 5 years after a severe fire. These were primarily *Rhizopogon* species; however, *Suillus* species were discovered in a subsequent greenhouse bioassay in which whitebark pine seedlings were planted in the burn soil (Trusty and Cripps 2011).

The inoculation of whitebark pine seedlings with *Suillus* fungi has the potential to increase seedling survival rates, especially in areas that may be devoid of ECM fungi. Even in areas that are not devoid of ECM fungi, establishing colonization on seedling roots prior to outplanting may give seedlings a jump-start during the critical initial establishment period (Ortega et al. 2004, Quoreshi et al. 2009). Also, as whitebark pine populations decline, closely associated fungi such as particular *Suillus* species will likely decline or shift in distribution (Mohatt et al. 2008, Karst et al. 2014). Therefore, inoculation may not only directly affect the health and survival of outplanted seedlings, but may indirectly encourage future seedling regeneration through the re-establishment of a healthy ECM community. Consequently, it is important that only native fungi be used in this process and that alien fungi and those found in commercial inoculum be avoided.

Monitoring

Over the past few decades, much effort has been put towards the restoration of whitebark pine through raising and outplanting seedlings. In order to validate these efforts, long term monitoring is necessary to understand the successes and failures of plantings and to fine tune restoration practices (Scott et al. 2011, Keane et al. 2012).

Whitebark pine is a slow-growing species and the widespread restoration initiative has only been implemented over the last few decades; therefore it is especially important to establish well-documented long term monitoring projects for both research and management purposes (Keane et al. 2012).

The monitoring of whitebark pine plantings are primarily performed by the National Forest Service, National Parks, and the Bureau of Land Management and reports vary in detail and in the data collected (Izlar 2007). Reports such as the *2015 Glacier National Park: Restoration Monitoring Report* (Asebrook and Hintz 2015) which documents whitebark pine plantings at 14 sites from 2000-2015 are invaluable in tracking planting prescriptions and site conditions in relation to seedling survival and can greatly aid land managers in improving the overall efficacy of plantings. Individual research efforts that document and monitor plantings have also proven useful in guiding planting prescriptions. Izlar (2007) conducted the largest monitoring project of whitebark pine plantings to date which encompassed approximately 115,000 seedlings across many sites and she found that overall, long term survival was low (38% for years 3-15). Mellman-Brown (2005) also reported low long term survival rates (14% by the 8th year) of over 2,000 whitebark pine seedlings outplanted at treeline in Montana and Wyoming. Lonergan et al. (2014) monitored almost 1,000 whitebark pine seedlings planted in Waterton Lakes National Park and found that overall survival was 69% 2 years after planting, and 47% 3 years after planting, and in this case survival was improved by planting on prescribed burns with microsites and by inoculation of seedlings prior to outplanting (Cripps et al. 2014).

The Power of GIS

One monitoring strategy that is not widely utilized in the restoration of whitebark pine is the spatial documentation and analysis of plantings. The use of advanced GIS concepts and modeling allow for better planning, for long term data collection, and for subsequent analysis. The majority of studies that document whitebark pine plantings use GPS technology to record the centers of study plots or the beginning and ends of transects so that seedlings can be relocated in future monitorings (Izlar 2007, Lonergan et al. 2014, Asebrook and Hintz 2015). However, none of these studies have incorporated the documentation of individual seedlings across a planting site in order to explore spatial patterns in survival and health using GIS software.

Though time consuming, the process of recording individual seedling locations utilizing handheld GPS technology results in a unique and invaluable dataset. Attaching recorded health data to seedling points in GIS software can reveal spatial patterns in health and survival that would go unnoticed when data are only recorded and presented in list form. Spatial tagging has been used to monitor outplanting and natural regeneration of seedlings in various habitat types for: wetland restoration of tree and shrub species in the southeastern U.S. (Freeland et al. 2010), tracking natural regeneration and survival of *Acacia* seedlings in Kenya (Wahungu et al. 2011), documenting tamarack and red maple occurrence in a bog in Ohio (Miletti et al. 2005), and spatially examining American chestnut seedling habitat in Mammoth Cave National Park (Fei et al. 2010). These methods have proven useful in some cases for determining the cause of some seedling

mortality and hold endless possibilities for monitoring restoration projects in unique ways.

Exploring seedling mortality in relation to planting variables in a spatial format has great predictive value for land managers. If spatial patterns of seedling survival correlate with planting variables, results could be used in choosing suitable future planting sites and in predicting future survival on specific sites. Variables that can be explored spatially in relation to seedling survival include (but are not limited to): slope, aspect, topographic wetness index (TWI), solar radiation, soil type, and planting density.

Restoration of Whitebark Pine in the Beaverhead-Deerlodge National Forest

The Beaverhead-Deerlodge National Forest (BDNF) lies in the Greater Yellowstone Ecosystem (GYE) where whitebark pine covers approximately 10% or 2.5 million acres of the landscape (GYCC 2011). Unfortunately, over 40-50% of stands in the GYE have experienced some level of canopy mortality largely due to mountain pine beetles or white pine blister rust and 95% of all stands exhibit mountain pine beetle activity (Goetz et al. 2009, McFarlane et al. 2010).

Whitebark pine occurs on approximately 15% or 150,000 acres of the Beaverhead-Deerlodge National Forest and 90,000 of these acres are classified as whitebark pine dominant stands (GYCC 2011). Restoration scores for whitebark pine stands based on canopy damage and stand structure indicate that 30% of whitebark pine in the BDNF has high restoration priority, which is the second highest level out of 8 national parks or forests in the GYE behind Yellowstone National Park (GYCC 2011).

Using aerial survey methods, McFarlane et al. (2010) classified the Gravelly Mountain range of the BDNF as having extreme WPBR mortality and moderate to high MPB mortality.

The Eureka Basin fire of 2013 began by lightning strike and burned over 4,000 acres of the Eureka Basin area in the southwestern portion of the Gravelly Mountain range (BDNF 2013). The majority of the burned area consisted of mixed whitebark pine stands which contained dead whitebark pine snags from MPB and WPBR kill; approximately 44% of the burn was classified as moderate-severe (Olson 2015). This large burn of whitebark pine habitat afforded land managers the opportunity to initiate a restoration planting program to fulfill a goal of the BDNF Forest Plan of 2009 to "promote regeneration of whitebark pine on approximately 45,000 acres, largely through the use of fire" (Olson 2015).

Over the next several years, land managers of the BDNF plan to plant 240,000-400,000 whitebark pine seedlings on 800-1,300 acres of the Eureka Basin burn, though this is contingent on funding (Olson 2015). Thus far, two whitebark pine plantings have occurred on the burn. The first planting, the focus of this study, was initiated in late June of 2015, soon after snowmelt when the site became accessible; approximately 36,000 whitebark pine seedlings were planted on a 107-acre planting unit. In early June of 2016, another 30-40,000 seedlings were planted on a 123-acre planting unit, a site that is not the focus of this study. Currently, the BDNF does not have a monitoring plan to follow up on current and future plantings of whitebark pine seedlings. Documentation and long term

monitoring of plantings could be very useful in planning future whitebark pine restoration in this area.

Research Objectives

This research project is part of a large-scale long term planting project to restore whitebark pine on several hundred acres of the Eureka Basin burn of the BDNF in SW Montana. The long term goals are to determine how seedling survival is affected by several planting variables including colonization by ectomycorrhizal *Suillus* fungi, slope, aspect, microsite type and position, and topographic wetness index (TWI, or potential soil moisture). Within the time frame of the current study, the objectives are 1) to explore the effects of planting variables on the early survival of seedlings and discuss implications for long-term survival, and 2) to develop a monitoring regime facilitated by spatial analyses through the use of GPS and GIS technology.

Although efforts are being made to restore whitebark pine across its entire range, this is the first study to utilize GPS and GIS technology to monitor individual whitebark pine seedlings across the landscape. This type of technology creates a unique spatial data set that can be used to explore whitebark pine seedling survival in new and valuable ways. The BDNF plans to continue restoration of the Eureka Basin Burn by planting whitebark pine on an additional 800-1,300 acres over the following years (Appendix A, Olson 2015). In 2016, the GPS/GIS methods developed and applied (to the 2015 planting) were also applied to the second annual planting (2016) on the burn; hopefully, the monitoring of both sites as well as the spatial documentation of future plantings will

continue in the coming years. This kind of monitoring not only documents seedlings for long term tracking, but can have predictive value for future planting sites.

Materials and Methods

Study Area Description

The study site is in the Eureka Basin which lies in the Gravelly Mountain Range in Madison County, in southwestern Montana, part of the BDNF (Figure 3.1), which is included in the Greater Yellowstone Area (GYA). The Gravelly Range is flanked by the Snowcrest and Greenhorn ranges to the west and the Madison Valley and the Madison Range to the east. Due to the cold, semi-arid climate, only 47% of the National Forest land in the Gravelly and Snowcrest ranges is covered by timber (Hamlin and Ross 2002). The remainder consists of large expanses of land above treeline and of high intermountain valleys (Cooper et al. 1997). Whitebark pine is often the dominant tree species above 8,500 ft, but it also occurs at lower elevations in the upper subalpine mixed with Engelmann spruce, subalpine fir, lodgepole pine, and limber pine. In recent years, whitebark pine populations have experienced heavy mortality due to mountain pine beetles and white pine blister rust.

In August and September of 2013 over 4,000 acres of the Eureka Basin area burned (Figure 3.1) due to a fire originating from a lightning strike (BDNF 2013). The fire burned large expanses of mixed conifer stands dominated by whitebark pine, subalpine fir, lodgepole pine, and Engelmann spruce. The fire was fueled in part by dying and dead trees impacted by beetles and rust. A whitebark pine stand in the area which

contained blister rust resistant cone-bearing trees was designated as high-priority for protection, but eventually 75% of the stand was severely burned and a genetically unique seed source was lost (BDNF 2013, Mahalovich 2015). Approximately 44% of the burned area (about 2,800 acres) was classified as moderately to severely burned (Olson 2015). In 2015, 107 acres of the Eureka Basin Burn was designated by the BDNF as a restoration site for the planting of 36,000 whitebark pine seedlings.

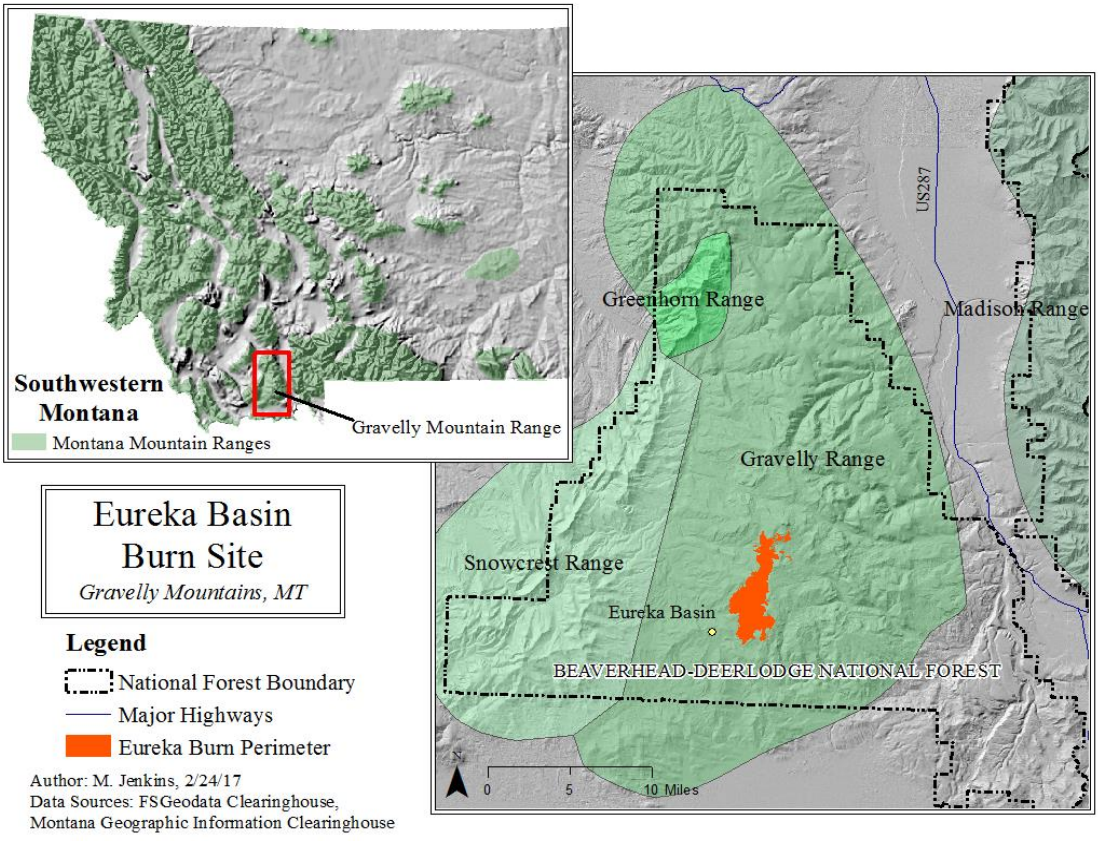


Figure 3.1: The Eureka Basin Burn site in southwestern Montana.

The 107 acre planting unit is SSW of Monument Hill and NNE of the West Fork Cabin at an elevation of approximately 2,774 m (9,100 ft) at latitude 44°50’39” N and

longitude 111°53’19” W. This site was selected by the BDNF for the planting of blister rust resistant whitebark pine seedlings because of the high elevation, moderate to severe burn conditions which cleared all over-story competition, and because of the presence of sufficient microsites and downed woody debris (Brennick 2015). The site was previously dominated by mature whitebark pine that was on average over 200 years old (Olson 2015). Mortality of all overstory and understory vegetation occurred across the entire planting unit due to the intensity of the fire; sparse, patchy understory 2-3 years after the fire included heartleaf arnica (*Arnica cordifolia*), fireweed (*Chamerion angustifolium*), Indian paintbrush (*Castilleja pudica*), and a few grasses (Figure 3.2). Minimal seedling regeneration was observed.

Figure 3.2: Planting unit conditions 2 years after the Eureka Basin fire in 2015.

The planting unit generally has a SW aspect and an average slope of 10°. The soil is loamy and is a complex of Adel-Garlet-Yellowmule families composed of sandstones, limestones, and quartzites deposited from landslides (Morrill 2000, Green 2007).

Approximately 75 liters of soil was collected along two 50-meter transects representative of the planting unit. For each transect, 3-4 liters of soil was collected every 5 meters to a depth of 15-20 cm. Samples were mixed and transported to the Montana State University Plant Growth Center (PGC) and placed in cold storage. A subsample was sent to Agvise Laboratories for an elemental analysis. The burn soil had a pH of 5.9, a cation exchange capacity (CEC) of 18.9 meq, organic matter content of 11.8%, and 12 mg kg^{-1} of nitrate; full results from the soil analysis are presented in Chapter 2, Table 2.1.

Water and climate data were obtained from the USDA Natural Resources Conservation Service (NRCS, https://www.wcc.nrcs.usda.gov/webmap_beta) using SNOTEL station 403, Clover Meadow, which is north of the study site in the Gravelly Mountains at an elevation of 8,600 ft (2,621 m) (NRCS 2017). The average annual temperature is 1 °C (33 °F); the average summer (Jul.-Aug.) temperature is 12 °C (54 °F) and the average winter (Dec.-Feb.) temperature is -8 °C (18 °F). The average annual precipitation accumulation by the end of the water year (beginning of Sept.) is 31” (79 cm) and the average snow water equivalent by the beginning of May is 18” (46 cm) (NRCS 2017). Annual precipitation accumulation for the 2015 water year was 25” or 6” below average (Figure 3.3); 2016 was similar at 27.5” or 3.5” below average annual precipitation. Annual snow water equivalent for the 2015 water year was 12” or 6” below average (Figure 3.3); the 2016 year was similar at 14” or 4” below average. Average annual soil moisture measurements from four depths are: 24% (4”), 41% (8”), 41% (20”), and 47% (40”). Annual soil moisture measurements for the 2015 water year were lower by 1% (4”), 1% (8”), 1% (20”), and 4% (40”); annual soil moisture measurements for the

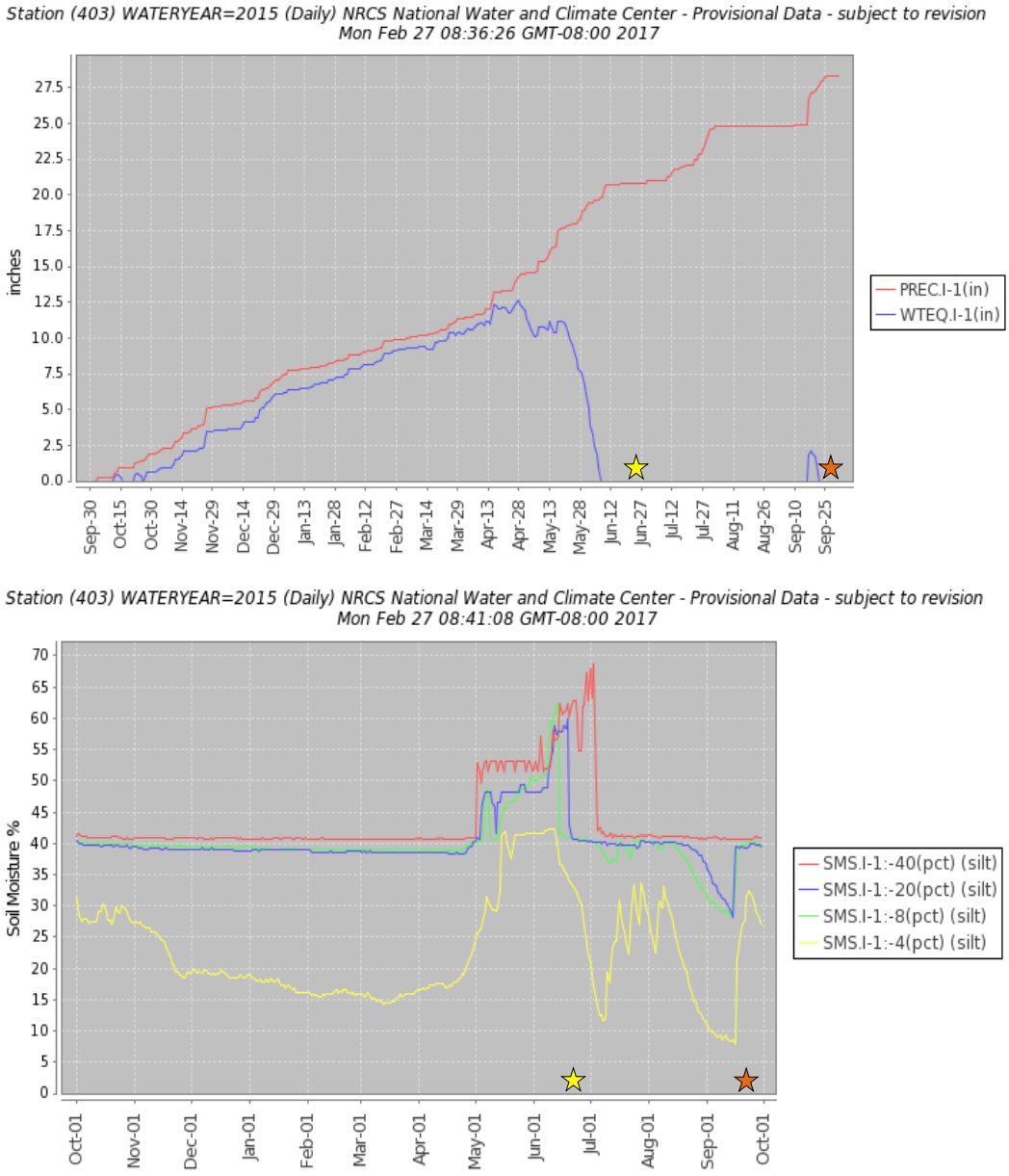


Figure 3.3: Precipitation accumulation and soil moisture graphs generated online for the 2015 water year (NRCS 2017). Top: Accumulated precipitation (in.) with total precipitation accumulation (red) and snow water equivalent (blue). Bottom: Soil moisture (%) measured at depths of 4" (yellow), 8" (green), 20" (blue), and 40" (red). Yellow stars indicate the 2015 BDNF whitebark pine planting date (June 23rd) on the Eureka Basin Burn. Orange stars indicate the first monitoring date (Sept. 26th, 2015) of seedlings.

2016 water year were greater by 1% (4”), and lower by 1% (8”), 3% (20”), and 2% (40”) (NRCS 2017). The total accumulation of precipitation from the time of the planting (June 23rd, 2015) to the first monitoring date (Sept. 26th, 2015) was approximately 7.5 inches (20 cm).

Seedlings

Approximately 36,000 potentially blister rust resistant whitebark pine seedlings were obtained by the BDNF for this planting project and they are from the same batch as seedlings used in the Chapter 2 greenhouse study. Seedlings were grown at the USDA Forest Service nursery in Coeur d’Alene, ID from seed collected from two seedlots, Crockett Lake (2,560 m) in the Gravelly Range and Moccasin Basin (2,800 m) in the Bridger-Teton National Forest (seed lot nursery ID tags: CROCKETTLAKE11 and WB03030092 respectively). Both seedlots are located in the Greater Yellowstone/Grand Tetons (GYGT) seed zone (Mahalovich et al. 2006). Seedlings were sown at the nursery by placing stratified non-scarified seeds in Ray Leach SC10 Super Cone-tainers (3.8 cm diameter, 21 cm depth; Stuewe & Sons, Inc., Tangent, Oregon) in a media of ground Canadian Sphagnum peat moss and composted Douglas fir bark (7:3). Seedlings were maintained for two growing seasons under standard nursery practices that included fertilization once or twice a week with $20N:7P_2O_5:10K_2O$ fertilizer (Peter’s Professional, The Scotts Company, Marysville, Ohio) and STEM micronutrients (soluble trace element mix, Peter’s Professional) (Eggleston 2010, Robertson 2015). Seedlings were finished with a $4N:25P_2O_5:15K_2O$ fertilizer and overwintered in freezer storage for vernalization from December 2014 to April 2015 (Robertson 2015). At this point seedlings were

packaged bare root in plastic bags of 10 with 25 bags per box and were transported to the BDNF Forest Service Butte Ranger District in Butte, MT to thaw in a seedling cooler prior to outplanting.

Ectomycorrhizal Colonization of Seedlings

It was not possible to inspect seedlings for ectomycorrhizal colonization prior to the outplanting process, thus seedlings were assessed on site. Boxes of seedlings were transported to the 107-acre planting unit in late June of 2015 by the BDNF via trucks and ATVs. Approximately 800 seedlings were randomly selected just prior to planting at the field site. Each was examined for colonization by ectomycorrhizal fungi and tagged; tags were color coordinated with colonization status (yellow tags for colonized seedlings, white tags for uncolonized seedlings) and each was labeled with an ID number. As in the greenhouse study, the vast majority of ectomycorrhizae observed were of the *Suillus* morphotype, characterized by a dingy white color and "hand like" branching mycorrhizae with white rhizomorphs (Treu 1990).

Planting

All 36,000 whitebark pine seedlings were planted on the 107-acre planting unit from June 23-25, 2015 by a planting crew contracted by the BDNF. Seedlings were planted with microsites for protection from wind, snow, and intense solar effects (McCaughey et al. 2009). Specifically, microsites were placed W-SW of seedlings to avoid prolonged exposure to direct afternoon sunlight (Brennick 2015). Seedlings were planted at a minimum of 6 feet apart with the intent of achieving 300 trees per acre

(Brennick 2015) and with the bottom needles touching the soil to avoid root exposure through settling of the soil (Figure 3.4).

Figure 3.4: A whitebark pine seedling planted with needles touching the ground.

The 800 seedlings tagged in the mycorrhizal assessment for this field study were planted by two crew members. Each researcher followed a particular planter and placed flags to mark each seedling as it was planted. This process resulted in two broad transects of randomly interspersed colonized and uncolonized seedlings (Figure 3.5). Transects were designated as “upper” and “lower” as the planting unit generally slopes to the SW. The upper transect runs partly along a ridgeline at a higher elevation that is more exposed to wind and is drier in comparison to the lower transect which is more protected from wind and less dry.

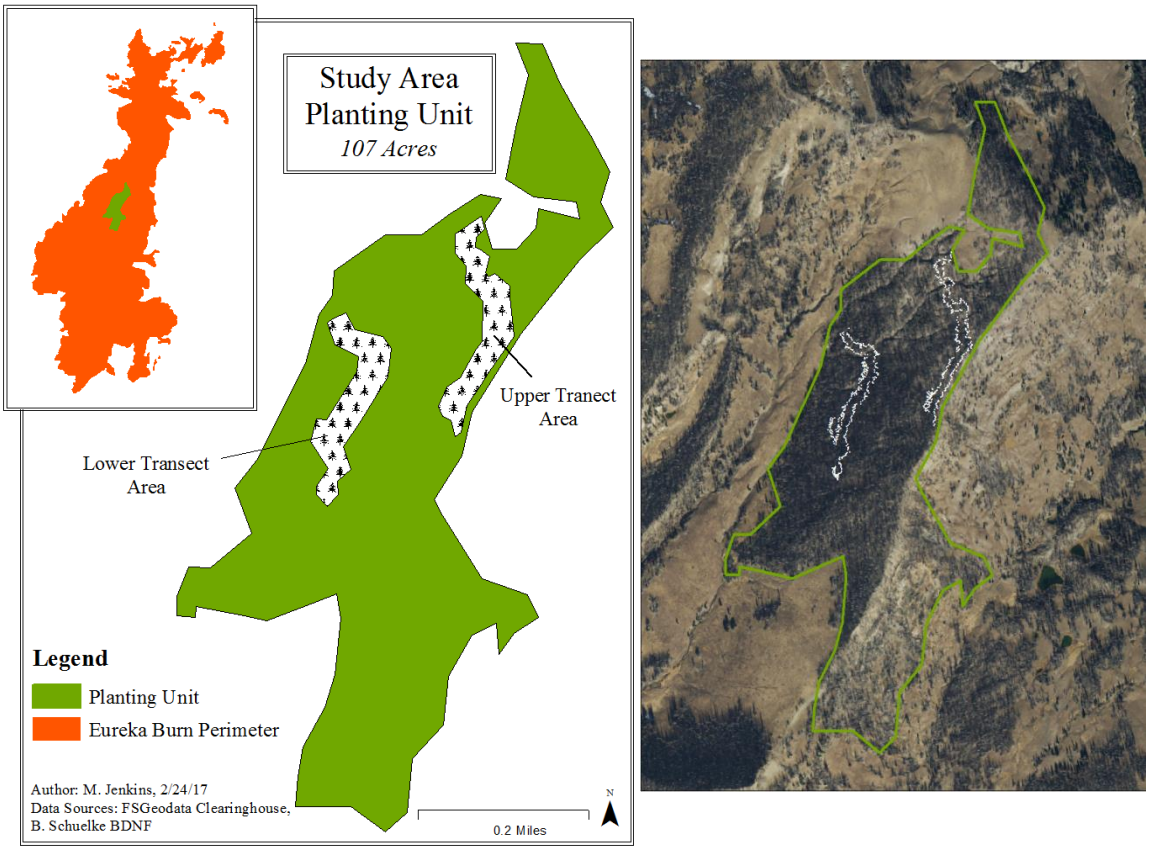


Figure 3.5: Left: Planting unit within the Eureka Burn with seedling transects. Right: Aerial photograph of site with planting unit boundary and seedling points.

Seedling Data

After seedlings were planted and flagged, vector data points were collected on the ground for each seedling using TerraSync™ software on a handheld Trimble GeoXH (©Copyright 2017, Trimble Inc.). Differential corrections were performed in the lab using GPS Pathfinder® Office Software (©Copyright 2017, Trimble Inc.) and resulted in an accuracy of 15 cm – 5 m. The resulting point data shapefile was in geographic coordinates and North American Datum 1983 (NAD83, Longley et al. 2015). In total, 378 seedlings that were colonized with ECM fungi and 371 uncolonized seedlings were located and given GPS coordinates after planting. There were 369 seedlings in the upper

transect and 380 in the lower transect, each containing colonized and uncolonized seedlings in randomized fashion.

The following data were recorded by hand for each of the 749 seedlings: ECM colonization status (colonized or not), microsite type, slope, aspect, and initial height. Slope was measured using a clinometer. Height was measured in centimeters from the cotyledon scar to the tip of the terminal bud (Haase 2008). Microsites were classified as logs, woody material (movable), snags (standing dead trees), and stumps (Figure 3.6).

Figure 3.6: Microsite classification. Row 1: log, woody material. Row 2: snag, stump.

Data were entered into an excel spreadsheet and attached to seedling vector points in ArcMap 10.4.1 (©Copyright 2016 Environmental Systems Research Institute, Inc.) using the Join function based on seedling identification number in the attribute table. Seedling points with attached data could then be used to visualize spatial distribution of variables tied to seedling parameter planting conditions. For example, seedling points could then be assigned a color based on colonization status and the distribution of colonized and uncolonized seedlings could then be visualized across the site (Figure 3.7).

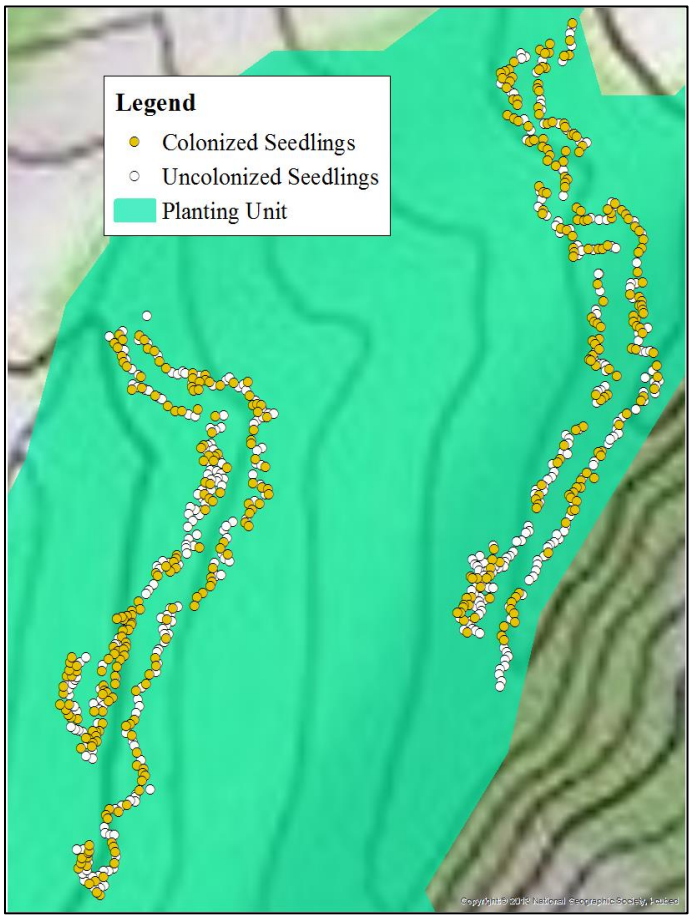


Figure 3.7: Visualization of the colonization status of outplanted seedlings using joined data in ArcMap software.

Monitoring Seedling Health

On September 26th, 2015 (approximately 3 months after planting) initial health and survival assessments were performed for all seedlings. Maps generated from seedling data points in ArcMap and microsite types were used to locate seedlings; seedling ID numbers were confirmed by the labeled tags if still present on seedlings. Health ratings were defined as: seedling dead (0), less than 25% of needles alive (1), 25 – 50% of needles alive (2), 50 – 75% of needles alive (3), 75 – 100% of needles alive (4). Researchers observed that some seedlings were buried in soil carried by water runoff that was piling up against microsites downhill of the seedlings. Therefore microsite position in relation to the seedling position was recorded and classified as: uphill, downhill, or side by side. Seedlings that could not be found or that had been pulled up by animals or buried in soil by runoff were recorded as such. Health and survival were monitored and recorded for all seedlings again on September 10th, 2016 (14 months after planting), using the same methods and health rating scale.

Statistical Analysis

Seedling height, which was measured at outplanting, was analyzed as a response to colonization status. Any differences were assumed to originate from a growth response in the greenhouse prior to outplanting. Analysis was performed using a simple regression model and data were displayed in boxplot format.

Seedlings were monitored twice: once 3 months after planting and once 14 months after planting. This monitors the early survival of the slow growing tree species, whitebark pine. In addition, seedling survival and health were explored graphically in

relation to various planting variables and by computing general statistics such as averages and standard deviations for data. Analyses were performed in R 3.1.2 statistical software (©2016 The R Foundation).

Health ratings were compared across colonized and uncolonized seedlings. For this comparison, seedlings that were recorded as dead from physical factors (such as being pulled up or getting buried in soil runoff) were removed. Spine plots were created for each monitoring year in order to determine the proportion of seedlings in each health category.

Spatial Analyses

Spatial analyses were performed in ArcMap 10.4.1 (©Copyright 2016 Environmental Systems Research Institute, Inc.). Seedling health and survival were explored in relation to several variables including transect, slope, aspect, microsite type and position, and Topographic Wetness Index (TWI). Seedlings recorded as dead were further separated into those obviously dead for physical reasons such as being buried (00) and those dead for no apparent physical cause (0) in order to compare planting variables for these two groups.

In order to calculate slope and TWI for this field study, a National Elevation Data (NED) 1/3 arc-second ArcGrid Digital Elevation Model (DEM), in geographic coordinates and North American Datum 1983, was downloaded from The National Map Viewer (http://viewer.nationalmap.gov/basic/). A DEM is a raster data set or a grid of cells that is representative of continuous elevation data with a measurement of elevation for each cell. This 1/3 arc-second data translates to approximately 8.7 x 8.7 meter cells,

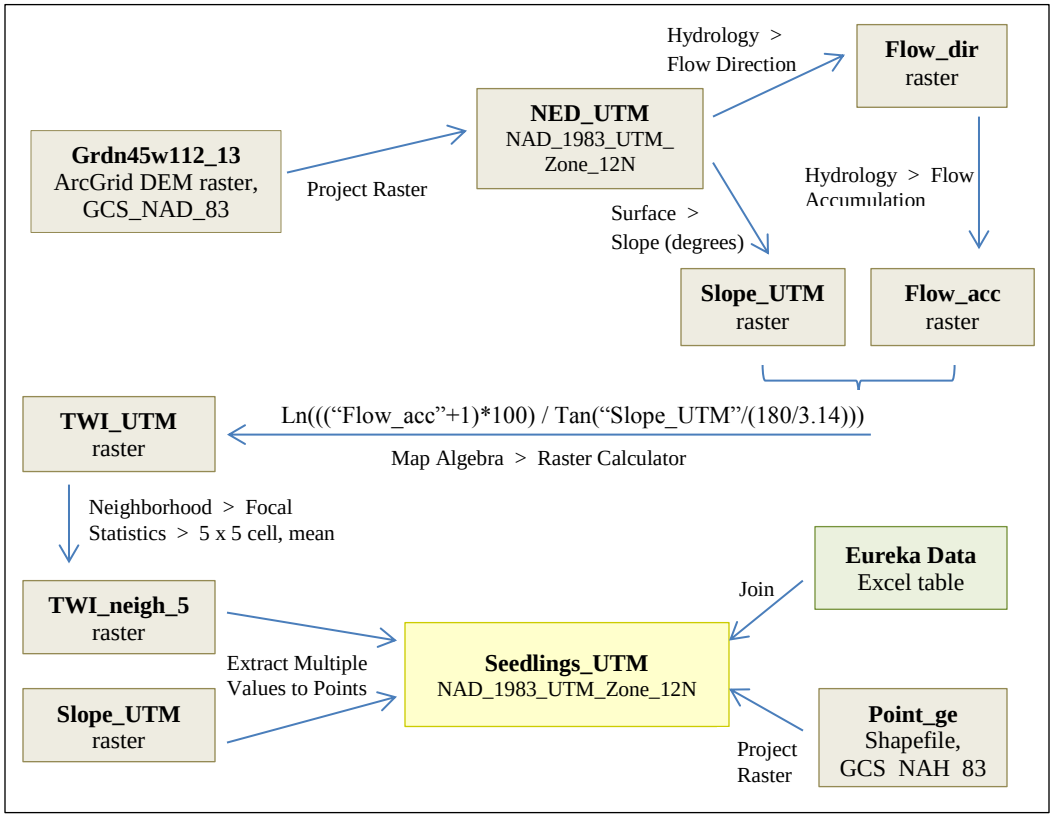
which calculates to a resolution of 1/17,000; therefore this is the scale at which data is accurate and at which it will be analyzed. At times, data are displayed at a smaller scale for visual purposes.

Slope. For the field study, the slope of each seedling planting point was determined in two ways: in the field using a clinometer, and from data extracted from a downloaded DEM. Measurements of slope recorded in the field were joined to the seedling point attribute table using the Join function in ArcMap. These measurements were used to create a raster of slope using the Conversion Tool, Point to Raster; cell assignment type was set to mean so that the slopes of two seedling points in a single cell would be averaged in order to assign a value to the cell. In the second method for determining slope, values were extracted from the DEM for each seedling data point using the Spatial Analyst Tool, Extract Values to Points. A map was created using each set of slope measurements in order to visualize the differences resulting from methodology.

Topographic Wetness Index. Topographic Wetness Index is a value calculated within a spatial interface that represents potential soil moisture in the field based on topography (Sörensen et al. 2006). Because the lower transect of seedlings appeared to be in a relatively wetter area than the upper transect, TWI values were calculated to explore any correlation with seedling health and survival values. The general formula for the calculation of TWI is defined as $\ln(a/\tan\beta)$ where a is the flow from the upslope area

through the sampled point and tanβ is the flow from the local slope of the point being sampled (Sörensen et al. 2006). Higher values indicate a greater wetness potential.

The manipulation of data within ArcMap in order to calculate TWI for each seedling point in this field study is represented in a flow chart (Figure 3.8) and is described hereafter. The initial NED DEM raster, (Grdn45w112_13) and the seedling point data set (Point_ge) were projected from geographic coordinates using the Project Raster tool to UTM Zone 12N which is the appropriate zone for the study site. This projection allows for the most accurate representation of data by minimizing distortion for the area represented (Longley et al. 2015). The DEM raster was then used to create a raster of local slope (in degrees) (Slope_UTM) using the Surface tool Slope. The DEM raster was also used to create a raster of flow direction (Flow_dir) using the Hydrology tool Flow Direction which was subsequently used to create a raster of flow accumulation (Flow_acc) using the Hydrology tool Flow Accumulation. These two raster data sets were then both entered into the Raster Calculator in the formula $\ln(a/\tan\beta)$, where a is the upslope area and tanβ is the local slope gradient, to produce a raster of TWI values for each cell (TWI_UTM). In order to account for the discrepancy between accuracy of datasets and uncertainty of assigning values to point data, a neighborhood analysis was performed which results in more a conservative estimate of TWI for each seedling. The TWI raster was entered into a Focal Statistics function to calculate mean TWI for each 5 x 5 cell neighborhood (TWI_neigh_5). Neighborhood TWI and local slope values (Slope_UTM) were extracted to seedling point data. An excel table including all field collected data (Eureka Data) was also joined to seedling point data (Seedlings_UTM).



Grdn45w112_13: downloaded DEM	**TWI_UTM**: a raster of calculated TWI values
NED_UTM: DEM projected to UTM Zone 12N	**TWI_neigh_5**: a raster of 5x5 mean TWI values
Slope_UTM: a raster of local slope	**Seedlings_UTM**: final point data with TWI values and data attached
Flow_dir: a raster of flow direction	**Eureka Data**: excel table of collected seedling data
Flow_acc: a raster of flow accumulation	**Point_ge**: original seedling vector points

Figure 3.8: A flowchart of data manipulation in ArcMap in order to calculate a TWI value for each seedling. Tan boxes are datasets used within ArcMap, blue arrows are functions used in ArcMap, the green box is an excel table, and the yellow box is the final resulting dataset.

Results

Seedling Height

On outplanting, seedlings colonized by ectomycorrhizal fungi were on average 1.12 cm or 21% taller than uncolonized seedlings ($p = <0.001$). Assumptions of normality and equal variance were satisfied. Data are displayed in Figure 3.9.

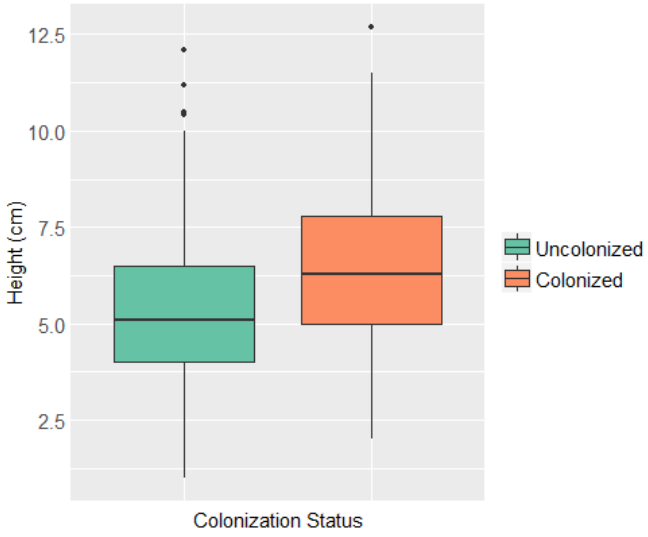


Figure 3.9: Boxplot of whitebark pine seedling height by ectomycorrhizal colonization status on outplanting.

Seedling Health

The health and overall survival of whitebark pine seedlings was very high in both monitoring periods (Figure 3.10). For the 2015 monitoring period the overall survival rate was 98%, and 90% of seedlings had the highest health rating of 4. For the 2016 monitoring period the overall survival rate was 94%, and 80% of seedlings had the highest health rating of 4. Of the 46 seedlings that had died by 2016, 39 (85%) were

recorded as dead due to a physical cause; of these seedlings, 35 were dead from being buried in soil carried by water runoff and 4 were pulled up likely due to animal activity.

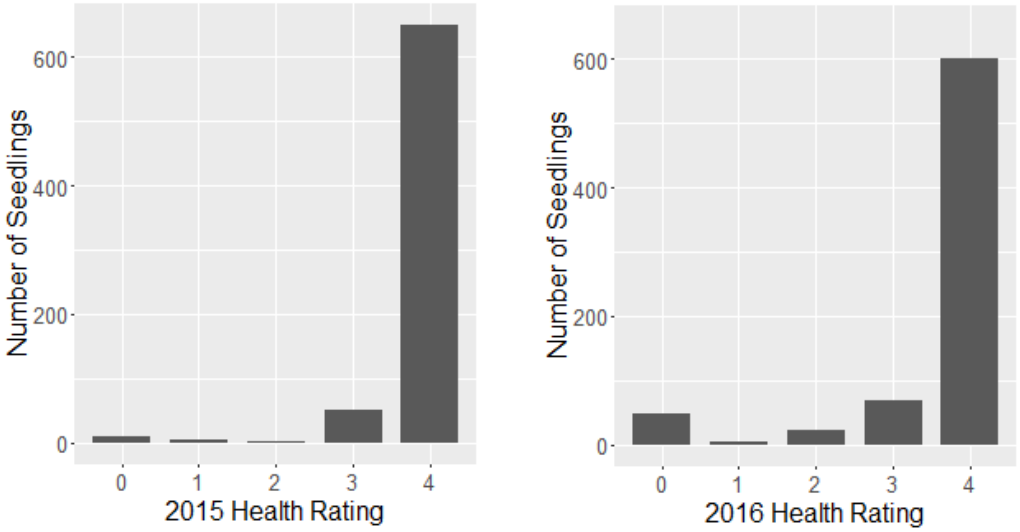


Figure 3.10: Health of whitebark pine seedlings at the 2015 and 2016 monitoring periods: zero indicates dead seedlings and 4 is the highest health rating.

Ectomycorrhizal Colonization and Seedling Health

After excluding seedlings that were dead due to obvious physical causes, seedling health ratings were plotted against ectomycorrhizal colonization status in spine plots for both monitoring periods (Figure 3.11). In 2015, after excluding seedlings that were dead due to obvious physical causes, the overall survival rate was 99% and 93% of seedlings had the highest health rating of 4. Approximately 9% of uncolonized seedlings and 4% of colonized seedlings had a health rating of 3. Seedling mortality was very low and no seedlings were given a health rating of 1 or 2. In 2016, after excluding seedlings that were dead due to obvious physical causes, the overall survival rate was 99% and 87% of

seedlings had the highest health rating of 4. Approximately 12% of uncolonized seedlings and 7% of colonized seedlings had a health rating of 3. Seedling mortality was very low and very few seedlings were given a health rating of 1 or 2. Data are presented in Table 3.1.

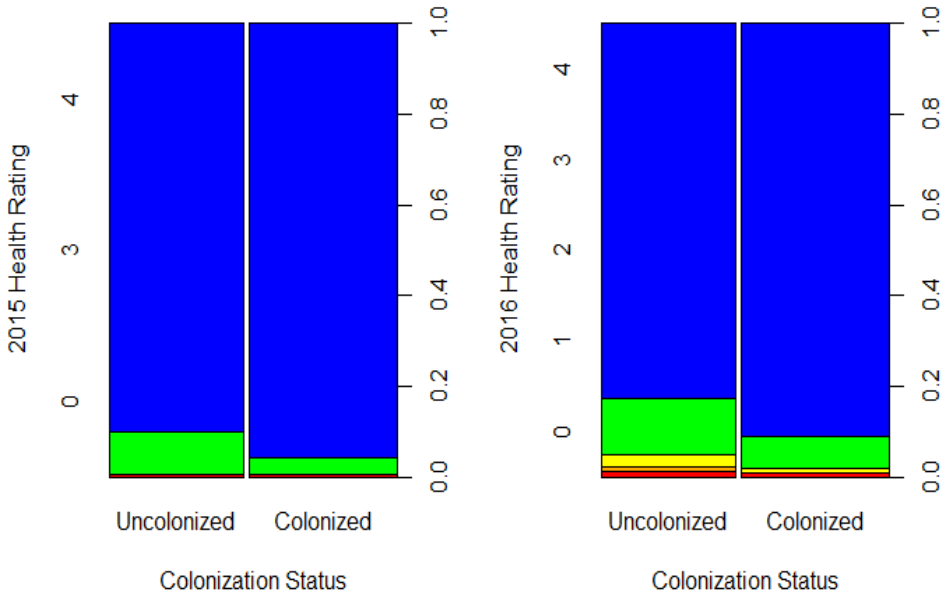


Figure 3.11: Spine plots of whitebark pine seedling health ratings by ectomycorrhizal colonization status for each monitoring period.

Table 3.1. Health ratings of whitebark pine seedlings by ectomycorrhizal colonization status for each monitoring period with 4 as the highest health rating.

	Colonization Status	Health Rating				
		0	1	2	3	4
2015	Uncolonized	2	-	-	30	289
	Colonized	2	-	-	13	340
2016	Uncolonized	4	3	9	40	269
	Colonized	3	0	4	25	325

Spatial Analyses

Seedling health ratings were mapped across the planting site (Figure 3.12). In total, 46 seedlings were recorded as dead with 40 in the lower transect and 6 in the upper transect. The majority of seedlings were dead due to obvious physical reasons such as being buried or pulled up (health rating = 00); only 7 seedlings were dead for reasons that could not be attributed to an obvious physical factor (health rating = 0, Figure 3.12).

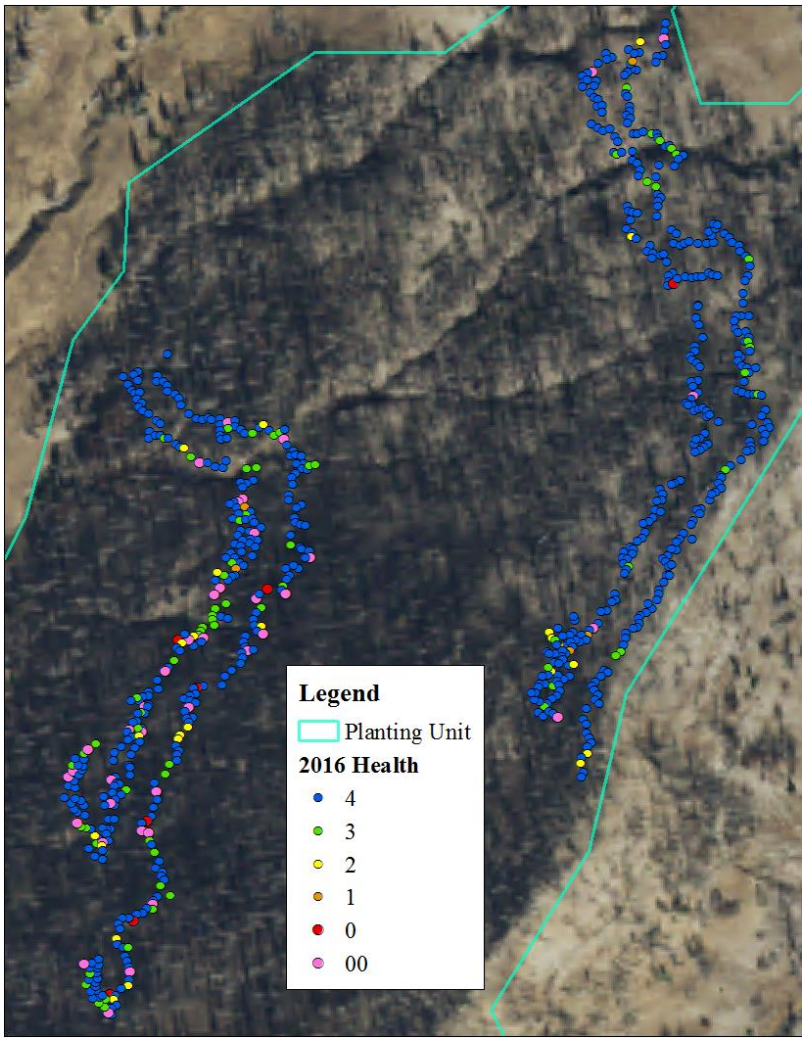


Figure 3.12: Health status of all seedlings from the 2016 monitoring period.

Visually, there is an apparent difference in seedling health in the upper and lower transects. Results from a Welch's two-sample t-test show that there is strong evidence against the null hypothesis that the average health rating of seedlings in the upper and lower transects is equal ($t = 6.24$, $p < 0.001$, $df = 577$). It is estimated that the average health rating of seedlings in the upper transect is 0.47 more than the average health rating of seedlings in the lower transect (95% CI of 0.32 to 0.62).

Slope. Of the two maps that were created utilizing different methods to measure slope, the map created using slope measurements recorded in the field (Figure 3.13, bottom left) captures more variation than the map created using slope measurements extracted from the DEM (Figure 3.13, top left). The steeper slope values indicated by the grey cells in the magnified box of field measured slope align with a drainage that runs through the site that the DEM did not capture. It is important to note that slope was only measured in the field at each seedling point, therefore the raster created from field slope measurements only includes cells in which the seedling points fall. This was overlaid on the raster of DEM derived slope measurements so that only the cells around seedling points change between the two maps.

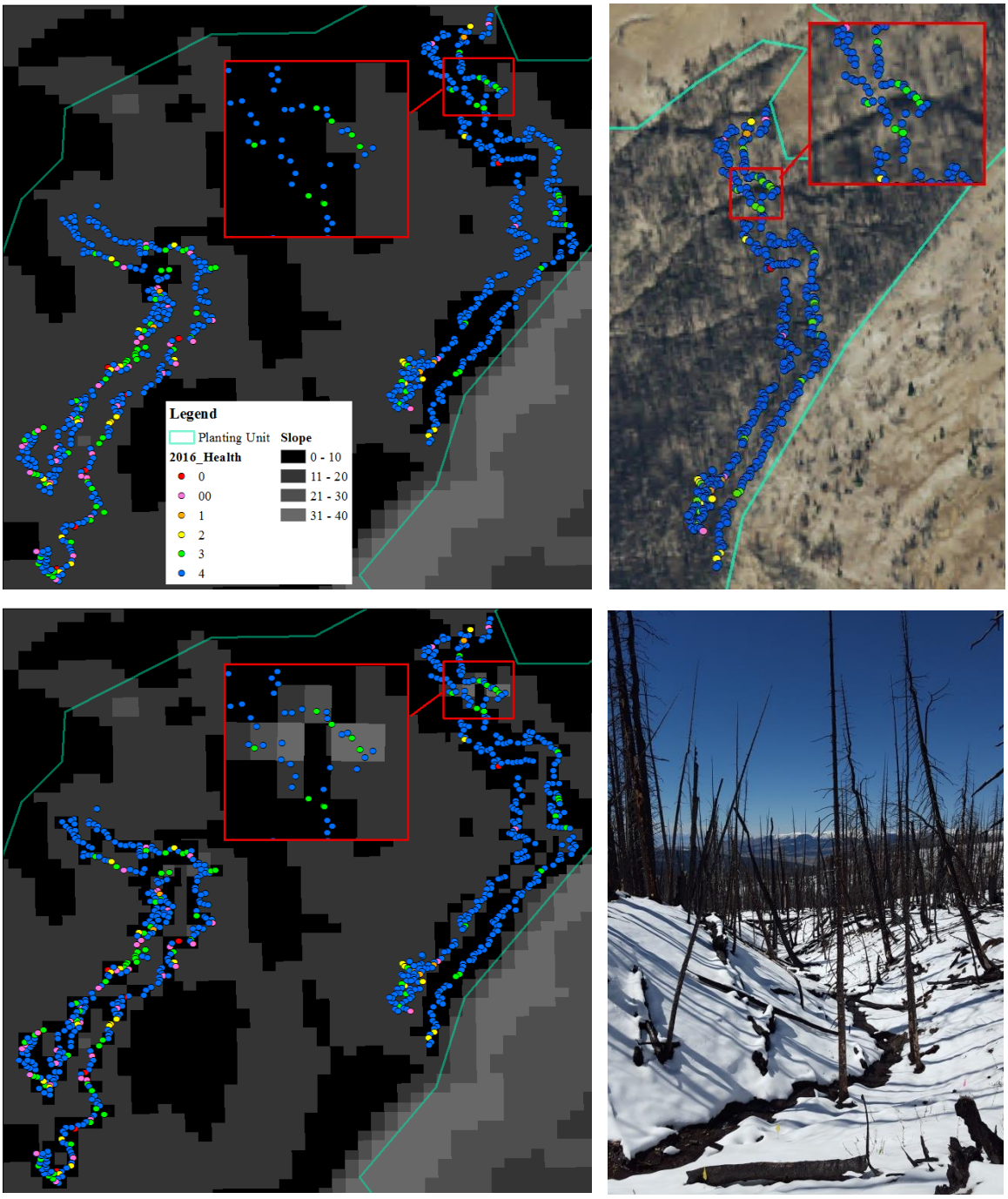


Figure 3.13: Top left: Map created using slope measurements derived from a DEM. Bottom left: Map created using field measurements of slope converted to a raster. Top right: An aerial photograph depicting fine scale land features including a small local drainage. Bottom right: A photo of the drainage running though the planting site.

Slope values from each method were averaged for each seedling health category (Table 3.2). Overall, averages were similar across methods except for the lower health ratings where there were fewer seedlings.

Table 3.2. Average slope values derived from each method across seedling health ratings.

Health Rating	n	Average Slope (°) (field measured)	Average Slope (°) (DEM extracted)
4	588	11	11
3	68	13	12
2	23	11	13
1	6	17	11
0*	7	9	14
00**	37	11	13

* seedling mortality not due to an apparent physical reason
** seedling mortality due to an apparent physical reason

Slope values from each method were also averaged across seedling health ratings for the upper and lower transects; averages were dependent upon method (Table 3.3). When comparing the field measurements of slope, the upper transect had a slightly higher average slope. When comparing the slope measurements extracted from the DEM, the lower transect had a higher average slope.

Table 3.3. Average slope values derived from each method for each transect.

Transect	n	Average Slope (°) (field measured)	Average Slope (°) (DEM extracted)
Upper	356	12	9
Lower	373	11	14

Microsite. Seedling mortality was explored in relation to microsite type and position. Microsites were mostly placed side by side or downhill of planted seedlings on the mostly SW sloping planting site. At times this appeared to result in seedlings being buried in soil moved by runoff piling up against the microsite (Figure 3.14). The majority of dead seedlings were in the lower transect, and these were planted with a variety of microsites (Figure 3.15). Overall, a majority of seedlings were planted with woody material (284), snags (252), and logs (179), and only a few were planted with stumps (15); seedling mortality by microsite type was 7%, 3.5%, 7%, and 13% respectively after 14 months (Table 3.4).

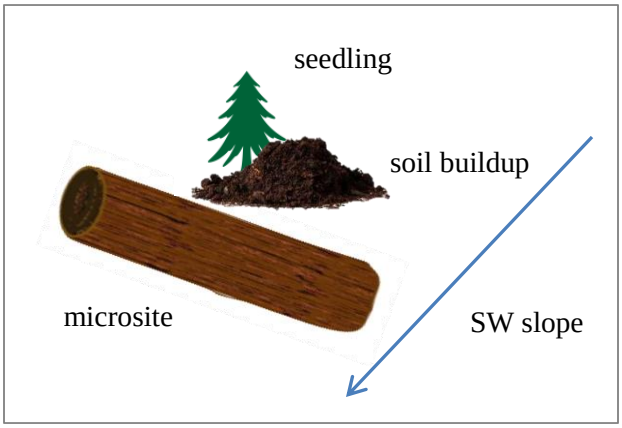


Figure 3.14: Left: Diagram of a soil deposition against a microsite that is placed downhill of a seedling. Right: In some cases, if the microsite is downslope, soil deposition from surface runoff can bury seedlings.

Table 3.4. The number of dead seedlings out of the total number of seedlings planted in each microsite type and its location relative to the seedling. Symbols are a legend for Figure 3.9 presented below.

	Woody Material	Log	Snag	Stump	Total
Uphill	1 of 27 (▲)	0 of 14	0 of 56	1 of 4 (▲)	2 of 101
Downhill	12 of 98 (●)	2 of 55 (●)	5 of 65 (●)	0 of 3	19 of 221
Side by side	7 of 159 (■)	11 of 110 (■)	4 of 131 (■)	1 of 8 (■)	23 of 411
Total	20 of 284	13 of 179	9 of 252	2 of 15	

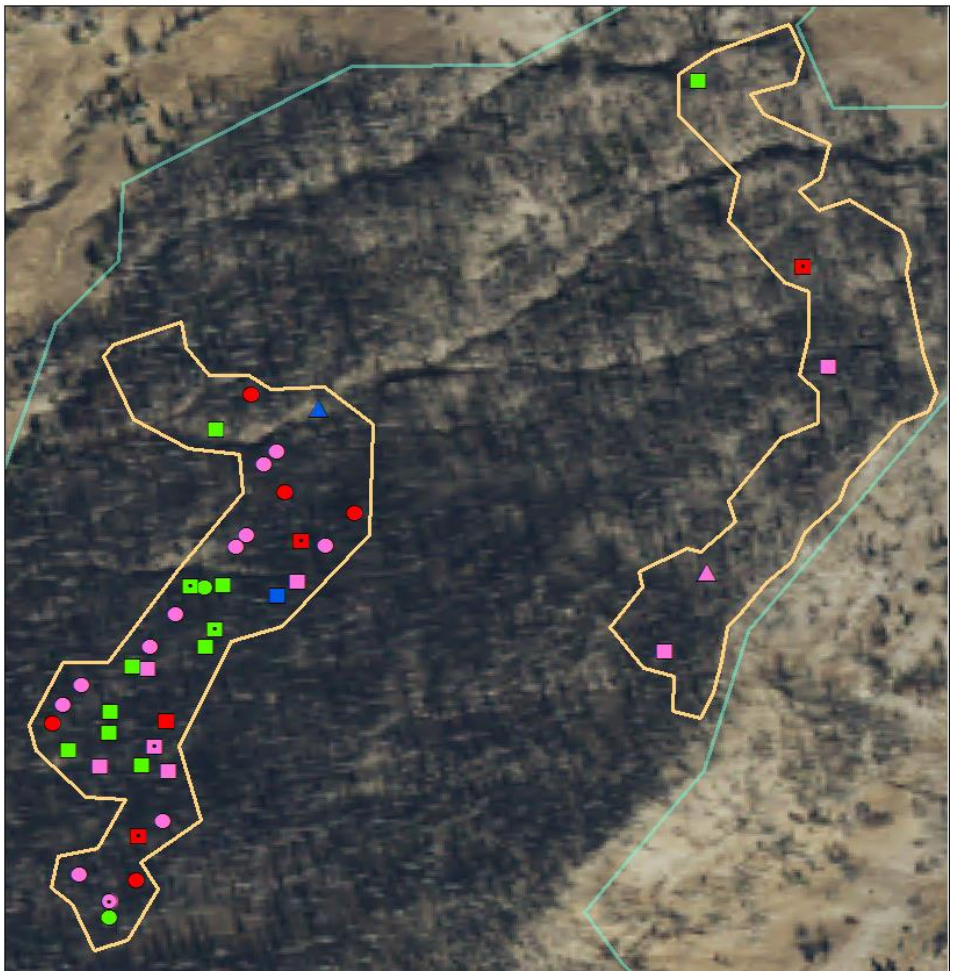


Figure 3.15: A map of dead seedlings in upper (right) and lower (left) transects symbolized by microsite type and position (legend in Table 3.4). A black dot inside a symbol represents mortality due to non-physical factor (n=7).

Topographic Wetness Index. Average 5 x 5 cell topographic wetness values are symbolized by color in Figure 3.16. Yellow cells in the TWI color raster, which indicate a higher potential for soil moisture, align with drainages captured in the aerial photo view of the planting unit. Overall it appears that the lower transect of seedlings (left) has higher TWI values, indicating a greater potential for wetness.

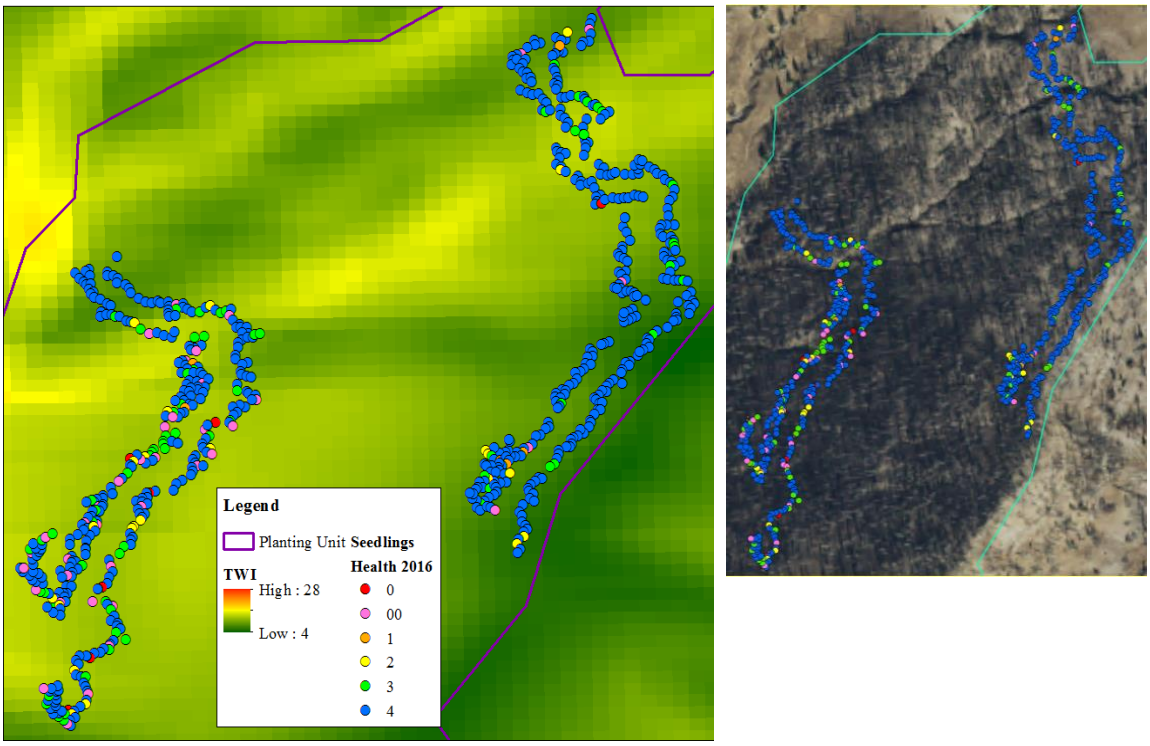


Figure 3.16: Left: A map of TWI and seedling health. Right: An aerial photo of the planting unit. Drainages in the aerial photo align with TWI patterns.

When explored graphically, the lower transect has a greater frequency of high TWI values in comparison to the upper transect (Figure 3.17). The average TWI of seedling points in the lower transect is 8.96 ± 0.52, and values range from 7.33 to 9.89.

The average TWI of seedling points in the upper transect is 7.85 ± 0.87, and values range from 6.20 to 9.81.

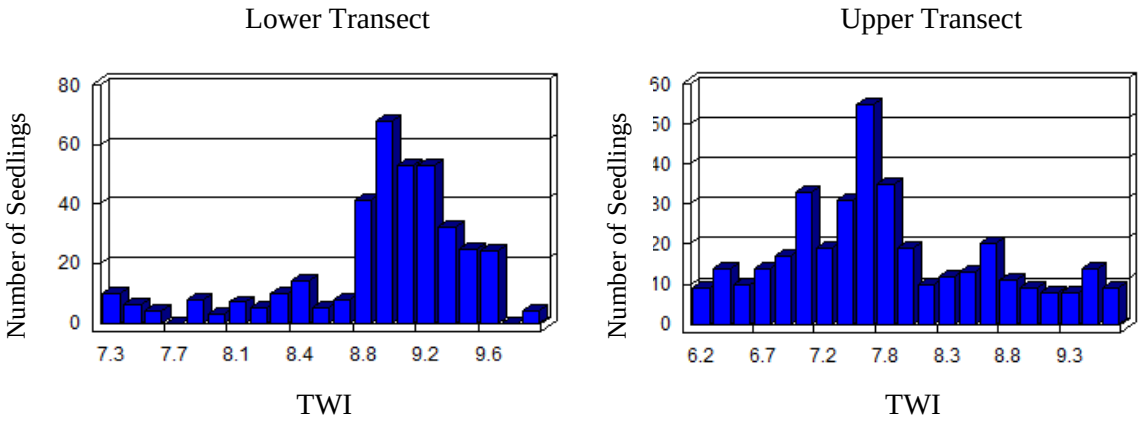


Figure 3.17: Frequency distributions of TWI values for seedlings by transect.

It was not possible to statistically correlate TWI with seedling health due to the uneven numbers of seedlings in health categories. A boxplot of data shows that boxes have unequal spreads and whiskers (Figure 3.18). Based on averages, it is unclear whether there is a trend between seedling health and TWI (Table 3.5). Dead seedlings had the highest average TWI of 8.93 when compared to other health ratings, but this was only slightly higher than for seedlings with a health rating of 3 (8.79). Dead seedlings in the lower transect had an average TWI of 9.11 (n=40) and dead seedlings in the upper transect had an average TWI of 7.73 (n=6).

Table 3.5. Average TWI values and standard deviations by seedling health ratings from 2016.

Health Rating	Average TWI	Standard Deviation	Seedling Count
0	8.93	0.60	46
1	8.27	0.88	6
2	8.49	0.89	23
3	8.79	0.74	71
4	8.33	0.92	596

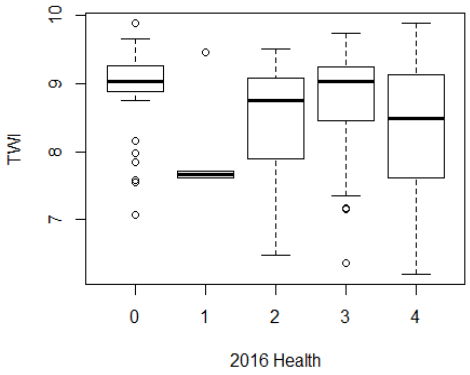


Figure 3.18: A boxplot of TWI by seedling health for 2016.

Discussion

The goal of this study is to examine how the health and survival of whitebark pine seedlings planted for restoration purposes on the Eureka Basin burn in the Gravelly Mountains of SW Montana are affected by particular biotic and abiotic factors across the landscape. These seedling-level variables include: root colonization by suilloid ectomycorrhizal fungi, microsite type and location relative to seedlings; slope, and topographic wetness index (TWI). This initiates a long term study with the ultimate goal of directly improving seedling survival with colonization by suilloid fungi and/or by providing information to land managers that can influence subsequent plantings as to where and how seedlings are planted. Even small increases in seedling survival contribute to the validation of the extensive efforts that go into the production of whitebark pine seedlings for restoration.

As a first step, methods were developed for assessing ectomycorrhizal colonization of seedlings in the field prior to planting. To monitor how this and other

variables affect seedling survival across the landscape of the burn, GPS data for individual seedlings were used in ArcMap; this resulted in unique spatial datasets that allowed individual seedlings (with their attached data) to be followed over the course of the current study, and that will aid in future assessments. Early results for monitorings at 3 and 14 months are presented in this thesis and future monitoring will provide additional data.

Seedling Survival

First year survival rates were high for whitebark pine seedlings planted on the Eureka Burn. Overall survival for the 2016 monitoring period was 94%. This is somewhat comparable to the first year survival rate for several other whitebark pine outplantings. Lonergan et al. (2014) reported a first year survival rate of 95% for almost 1,000 seedlings planted under a variety of treatment combinations in Waterton Lakes National Park. Izlar (2007) reported an average first year survival rate of 74% for approximately 114,000 seedlings planted across a variety of sites. First year survival rates for whitebark pine seedlings planted on 14 sites in Glacier National Park (GNP) range from 30-99% with an overall first year survival rate of 72% (Asebrook and Hintz 2015). One major discrepancy between the GNP plantings and the BDNF planting of the current study is that they differ greatly in scale. The GNP plantings are being carried out on a much smaller scale, with a maximum of 1,300 seedlings planted on a site while the BDNF aims to plant 30-40,000 seedlings on 100-125 acres per planting (Asebrook and Hintz 2015, Olson 2015).

Survival rates of whitebark pine seedlings tend to decrease rapidly after the first year, so long term monitoring is necessary to determine the success of plantings. Cripps et al. (2014) reported that although the first year survival rate of whitebark pine seedlings planted in Waterton Lakes National Park was 95% (Lonergan et al. 2014), survival dropped to 69% in year two and to 47% in year three. Izlar (2007) reported that first year survival rates dropped from 74% to 38% in years 3-15. Glacier National Park plantings have resulted in various longer term survival rates of 31% and 41% for 14- and 13-year old plantings, respectively, and a 65% survival rate for seedlings on a 15-year old planting site (Asebrook and Hintz 2015). Future monitoring of whitebark pine seedlings on the Eureka burn study site will determine whether long term survival rates are comparable.

The majority of seedlings that experienced mortality after one year in the current study were dead due to an obvious physical cause (39 of 46, or 85%). If these seedlings are removed from the analysis, the overall survival rate would be 99%. Of the seedlings dead from a physical cause, 35 (90%) had been buried in soil deposited by water runoff that accumulated against microsites. McCaughey et al. (2009) reported that placing shelter objects upslope of seedlings can protect seedlings from soil deposition by redirecting water flow, however even when seedlings were buried, they often survived. In the current study, there are examples of partially buried seedlings that have survived thus far but longer term monitoring is necessary to determine the overall loss of seedlings due to soil deposition. Other studies also report that seedling mortality in the initial years after planting is largely due to physical factors, though this is mostly a result of animal

predation (Izlar 2007, Asebrook and Hintz 2015). Only 4 seedlings in this study were pulled up due to presumed animal activity.

Ectomycorrhizal Colonization of Seedlings

The effect of ectomycorrhizal colonization on the health and survival of whitebark pine seedlings was not analyzed statistically as the vast majority of seedlings (87%) had the highest health rating of 4 as of the 2016 monitoring. After excluding seedlings that were dead due to obvious physical reasons, the death of only 7 seedlings remained unexplained, 4 of which were uncolonized and 3 of which were colonized by suilloid fungi on outplanting. The primary difference in seedling health overall was that 12% of uncolonized seedlings were assigned a lower health rating of 3 in comparison to 7% of colonized seedlings assigned this health rating. Seedling survival was high after only one year and any effects of colonization will likely take longer to become apparent. For example, Lonergan et al. (2014) reported that inoculation of whitebark pine seedlings with *Suillus sibiricus* prior to outplanting in Waterton Lakes National Park did not exhibit an effect on seedling survival after one year when survival was still high (95%). However, seedling survival rates were increased by 6% in year two and by 11% in year three by inoculation alone (Cripps et al. 2014). In a 2012 planting at Divide Mountain in GNP inoculation of seedlings with *Suillus* several months prior to outplanting increased overall seedlings survival by 16% three years after monitoring; the increase in survival was apparently tied to other planting conditions as well, for example seedlings being planted on burned areas (Asebrook and Hintz 2015).

The results of the monitoring of a 2010 GNP planting at Divide Creek, in which a portion of seedlings were inoculated with *Suillus* fungi prior to outplanting, showed that inoculated seedlings tend to be taller (though not statistically significant) five years after planting. Inoculated seedlings tended to be initially taller at the time of outplanting and subsequently tended to grow more after being planted. From 2010-2015, inoculated seedlings grew on average from 9.90 cm to 12.98 cm (or 3.08 cm) and un-inoculated seedlings grew on average from 8.61 to 10.06 cm (or 1.45 cm; Asebrook and Hintz 2015). An increase in height can often but not always be directly correlated to a greater number of needles, which is indicative of photosynthetic capacity; however taller seedlings can be more susceptible to moisture stress and harsh weather conditions (Haase 2008, Grossnickle 2012). In the current study of the 2015 BDNF planting, colonized seedlings were significantly taller at the time of outplanting by 1.12 cm or 21%. In the greenhouse study portion of this thesis (Chapter 2), colonized seedlings were initially taller when received from the Coeur d'Alene nursery (data not presented); after being grown in an unsterilized burn soil mixture for 6 months, colonized seedlings on average had 27% greater biomass (Table 2.5) and 26% greater foliar nitrogen content (Table 2.7) than uncolonized seedlings. Future monitoring of seedlings at the field site will determine if this greater initial height of colonized seedlings tends to correlate with greater growth and overall survival.

It is important to consider the difference between 'inoculation' and 'colonization' of seedlings by ectomycorrhizal fungi. Inoculation does not always result in colonization of seedlings and efficacy is dependent upon the substrate, inoculation rate, and host

suitability (Davey et al. 1990, Brundrett et al. 2005, Rincón and Fernández-Pascual 2005, Cripps and Grimme 2011). This is the first study to assess the actual colonization status of whitebark pine seedlings prior to outplanting. Other studies have used ‘inoculation’ as the explanatory treatment, because it was not possible to assess actual colonization at planting and because destructive sampling after outplanting often is not possible (Lonergan et al. 2014, Asebrook and Hintz 2015). While often unavoidable, this can obscure the effects of actual colonization on seedling outplanting performance.

Planting Whitebark Pine on a Burn Site

While whitebark pine is historically tied to wildfire and has an advantage in pioneering burn sites due to its unique dispersal mechanism, natural regeneration is also dependent upon the distance to a seed source and on the health of that seed source. McKinney and Tomback (2007) found that as blister-rust damage increases within a whitebark pine stand, seed dispersal by nutcrackers is also likely to decrease. Leirfallom et al. (2015) found that natural regeneration on burn sites was sparse if more than 50% of the seed source was damaged or dead. It is now recommended that whitebark pine sites experiencing >20% blister-rust-induced mortality, >50% blister rust infection, or high mortality from pine beetles be replanted as natural regeneration might not occur (Keane and Parsons 2010, Keane et al. 2012). Additionally, studies show that the natural regeneration of whitebark pine is likely dependent on disturbance frequency; for example, although a single disturbance such as a fire may encourage regeneration, overlapping disturbances may have the opposite effect and regeneration may be low (Raffa et al. 2008, Larson and Kipfmueller 2010).

All whitebark pine seedlings in this study were planted on a burn and none were planted in adjacent unburned areas, therefore it was not possible to directly determine if planting on a burn is advantageous to seedling survival. However, other studies have reported on the advantages of planting on burns. For whitebark pine in particular, planting is recommended on recently burned sites to mimic the dispersal of its seed by the Clark's nutcracker, which often caches seeds on burns (Tomback et al. 1993, Keane et al. 2012). Additionally, burn sites offer areas where there is reduced competition and plenty of available microsites (Keane et al. 2012).

All of the aforementioned studies that explored the effects of inoculation of seedlings with *Suillus* fungi prior to outplanting simultaneously compared seedling survival across burned and unburned areas. Lonergan et al. (2014) found that when seedling survival was averaged across other planting variables, their overall survival rate on small prescribed burn areas (classified as non-lethal to moderate) was 70% in contrast to 51% on unburned areas. The highest third year survival rates (63%) were for seedlings planted on burns that were inoculated and planted with a microsite (Cripps et al. 2014). Izlar (2007) found that whitebark pine seedling survival was greatest on mixed-severity burn sites (52%), and was reduced on severe burn sites (41%), and on unburned sites (21%). Planting on burns has increased seedling survival on several GNP planting sites, and at Divide Mountain the highest survival rate of 90% was reported for seedlings inoculated and planted on burns; this was substantially higher than the 30% survival rate reported for un-inoculated seedlings planted on unburned areas (Asebrook and Hintz 2015). In a study that directly planted whitebark pine seed in the soil, germination and

survival rates were three times greater in recently burned plots than in unburned plots, and the average biomass of seedlings in a burned plot was 57% greater than that of seedlings in an adjacent unburned plot (Perkins 2015). The positive effects of planting whitebark pine seedlings on burns are mainly attributed to reduced competition, microsite availability, and nutrient status of soil (Lonergan et al. 2014).

Some studies have reported soil characteristics for unburned whitebark pine forests but we could find no information for soils from burned whitebark pine forests. One study which sampled soils from 8 whitebark pine stands in the GYE reported that the pH ranged from 4.56-5.9 and organic matter varied from 4.4-8.2% (Morrill 2000). In a review of several studies, Hansen-Bristow et al. (1990) report that pH values for whitebark pine forests range from 5.1-6.5, organic matter from 2.2-6.1%, and CEC from 5.3-21.2 meq (Hansen-Bristow et al. 1990). Keville et al. (2013) reported that soils collected from whitebark pine stands in the Pioneer Mountains of southwestern MT in the Beaverhead-Deerlodge National Forest contained less than 1 mg kg^{-1} nitrate (NO_3^-); several other studies have reported nitrate values less than 1 mg kg^{-1} for conifer stands in the Rocky Mountains which is considered as low (near detection limits) or limiting (Douglas et al. 2005, Kennedy et al. 2015, Trahan et al. 2015).

In the current study, the burn soil had a pH of 5.9, an organic matter content of 11.8%, a CEC of 18.9 meq, and a nitrate content of 12 mg kg^{-1}. Therefore in comparison to unburned forest sites, the burn soil from the current study site has elevated nitrate levels, a pH within normal range, and a high organic matter content, all of which can be characteristic of burn soils. Elevated nitrate levels are beneficial to seedlings in the

establishment period and a pH close to neutral (7.0) can be indicative of productive soils; if pH is too high or too low, nutrients can become insoluble and unavailable for uptake (Meurisse et al. 1991). High organic matter content would be expected because of the burned organic material present and can be indicative of a greater potential for soil nutrient and water retention as well as providing a substrate for the decomposition process (Page-Dumroese et al. 1991).

Spatial Analyses

The initial spatial representation of seedling health across the planting site revealed that seedlings in the lower transect experienced greater mortality and lower overall health (Figure 3.12), which was confirmed by statistical analysis. This could be due to bias attributed to particular seedling batches or particular planters; however, most early mortality could be attributed to physical factors related to microsite, slope, and possibly elevation, which correlated with potential soil moisture (TWI).

Slope and Aspect. Whitebark pine occurs on a variety of slopes and aspects (Hansen-Bristow et al. 1990) and seedling survival has been related to plot- and stand-level slope and aspect in several studies. Izlar (2007) found that site- and plot-level measurements of slope did not significantly affect seedling survival; however, based on personal observations from extensive monitoring, she recommends planting on sites with a 10-45° slope and not on flat ground. Scott and McCaughey (2006) reported that the 11th year survival of outplanted whitebark pine seedlings was greater on a ridge bench with a 9% (5°) slope than on an adjacent area with a 15% (9°) slope. In another study, naturally

regenerating whitebark pine seedlings grew better on gentler slopes when plot-level slopes varied from 20°-35° (Moody 2006). Slope and aspect were taken into consideration by land managers in the selection of potential planting sites on the Eureka Basin burn (Brennick 2015).

Seedling-level measurements of slope across a single site have not been related to survival rates or overall health in any of the previous studies. In the current study, seedling-level slope, which ranged from 0-45° in field measurements and 0-20° in DEM extracted measurements, did not have a clear effect on seedling health or survival 14 months after planting. The results for slope measurements were method dependent and field measurements appeared to be more accurate in comparison to DEM extracted measurements. It is important to note that the 1/3 arc second DEM raster cells translate to 8.7 x 8.7 m areas on the ground, or about 76 square meters. Comparison of maps created from the two methods to an aerial photograph of the site revealed that field measurements of slope captured features on a finer scale (Figure 3.13). However, whichever method was used there appeared to be no correlation of slope to seedling health for the slope range of the study after 14 months (Table 3.2).

The aspect of the current study site is mainly southwest to west facing. The early natural regeneration of whitebark pine seedlings tends to be greater on sun-exposed southern aspects (Arno and Hoff 1989, Moody 2006, Scott and McCaughey 2006), but the largest and best-formed trees are often found on north-facing slopes (Arno and Hoff 1989). Additionally, it has been shown that while early regeneration can be greater on warmer southern slopes, sapling recruitment and longer term survival appear greater on

northern slopes (Moody 2006). Aspect can affect many site factors including snow accumulation, soil properties, solar radiation, wind, and temperature (Hansen-Bristow et al. 1990, Morrill 2000). Future plantings across a variety of sites on the Eureka Burn will allow researchers to examine how site-level slope and aspect affect seedling health.

Microsite. After one year, microsite type did not appear to have a clear effect on seedling health and survival in the current study. McCaughey et al. (2009) recommend planting whitebark pine seedlings on the north side of stationary objects such as stumps, rocks, and large logs for shade and protection from physical stresses. Planting prescriptions recommend avoiding planting seedlings with snags, as eventually they are likely to fall and uproot or damage the seedling (McCaughey et al. 2009). In the current study 252 of the 730 seedlings (35%) for which microsite information were recorded planted with snags (standing dead). Long term monitoring can reveal if seedlings planted with snags experience higher mortality in the future. Microsite position appeared to have a slight effect on seedling survival in this study. When seedlings were planted uphill or side by side of microsites, in some cases water runoff deposited soil against the microsites which buried seedlings, as has been observed in previous studies (McCaughey et al. 2009). Again, future monitoring can help determine if soil deposition is a significant factor in seedling mortality.

Topographic Wetness Index. The current study planting site was selected by the BDNF because of its suitability for whitebark pine as determined by several variables. Sufficient moisture availability is critical for seedlings in the period immediately

following outplanting and for natural seedling recruitment (Tomback et al. 2001, Grossnickle 2012). Therefore, seedlings were planted as soon as the site was accessible in order to capitalize on soil moisture from snow melt as is recommended in planting prescriptions (McCaughey et al. 2009). Measurements from a nearby SNOTEL station reported that at the current study site, soil moisture ranged from 8-34% (at a depth of 4") in the three months following outplanting; at 8 and 20 inches it ranged from 28-41%. McCaughey (1990) reported that soil moisture values ranged from 3-42% at a depth of 2 inches (5 cm) from June-Oct. on a recently clearcut mixed whitebark pine forest in the Gallatin National Forest, MT. The total precipitation accumulation for the three months following planting at the current study site was approximately 7.5 in (19 cm) which is 2 inches (5 cm) below average. In one study, Perkins (2004) hypothesized that low whitebark pine seedling recruitment at one site was connected to low summer precipitation (19 cm, June-Sept.) and that high recruitment at another site was connected to higher summer precipitation (32 cm, June-Sept.). McCaughey et al. (1990) also hypothesized that the low germination rates of outplanted whitebark pine seed after one year were connected to low precipitation (6 cm, June-Sept.) and that higher germination rates another year were connected to higher precipitation (14 cm, June-Sept.).

Although whitebark pine seedling establishment is reliant upon adequate soil moisture, overall long term seedling survival tends to be higher on drier sites. One study showed that by the 9th year after outplanting, the survival of whitebark pine seedlings was greater on dry sites (86%) in comparison to that on moist sites (50%; Scott and McCaughey 2006). Another study found that the number of whitebark pine seedlings

establishing after the 1988 Yellowstone fires was highest on moist sites, but that overall survival was higher on dry sites (Tomback et al 2001). Perkins (2004) found that the survival of outplanted seedlings was lower on moist sites which were associated with sedges. The terms of ‘moist’ or ‘dry’ are of course relative within each of these studies and for various sites.

During the 2015 September monitoring, researchers observed that the upper transect area appeared much drier than the lower transect area. The lower transect was downhill of the ridge and in several places, water seemed to be concentrated in fine scale drainages and on flatter areas, so a topographic wetness index (TWI) was used to estimate the potential soil moisture at each seedling point. The average TWI was higher (8.96) for the lower transect in comparison to the upper transect (7.85), with higher numbers indicating a greater potential for soil moisture. The average TWI of dead seedlings in the lower transect was 9.11, which is higher than the average TWI for all seedlings in the lower transect. However, there was no significant evidence that seedling health was directly correlated to TWI overall. Future monitoring will be necessary to determine whether long term seedling survival correlates with TWI.

Measures of potential soil moisture have been related to the establishment of whitebark pine seedlings in several studies. Larson and Kipfmueller (2010) correlated the natural regeneration of whitebark pine with several biophysical site variables in 60 plots across 6 different mountain ranges. One measure included in their analysis was a topographic relative moisture index (TRMI), calculated as a sum of slope steepness, slope configuration, slope aspect, and slope position for each 0.1 ha plot. This measurement

was then used to explore the effects of topography on moisture availability at a stand level (Larson and Kipfmueller 2010). Results showed that there was no significant relationship between seedling (<2 cm diameter at ground level) density and relative TRMI, but that there was a significant negative correlation between sapling (≥2 cm diameter at ground level) density and relative TRMI (Larson and Kipfmueller 2010). This suggests that while seedling establishment may not be significantly correlated to topographic moisture availability, long term sapling establishment is greater on drier sites. In another study, Leirfallom et al. (2015) correlated whitebark pine seedling regeneration on large burns with various site characteristics that included topographic convergence index (TCI, equivalent to TWI); in this case natural seedling occurrence in burn sites was not significantly correlated with TCI. However, both of these studies that utilized topographically derived measurements of potential soil moisture to predict natural whitebark pine regeneration have been used at a plot- or stand-level (Larson and Kipfmueller 2010, Leirfallom et al. 2015). The current study is unique in that TWI was calculated across the entire site and can be correlated to individual seedling success. If TWI does correlate with seedling health, this could be a valuable tool for land managers to use when selecting future planting sites.

The calculation of TWI relies only on slope and is therefore somewhat limited in its applicability. Additionally, it is recognized that environmental conditions other than soil moisture are affected by topography and can have important impacts on microclimate and therefore seedling success. For example, it has been shown that cold air drainage flow in mountainous areas can have significant effects on local temperature (Novick et al.

2016). Variations in microclimates of planting sites could directly affect seedling establishment as well as indirectly by interacting with soil moisture.

Summary and Conclusions

For the large-scale planting of 36,000 whitebark pine seedlings on the Eureka Basin Burn in the Beaverhead-Deerlodge National Forest, the first year survival of the 800 seedling subsample was high overall (94%). A method for examining how seedling-level planting variables such as colonization by suilloid ectomycorrhizal fungi, microsite type and position, slope, and potential soil moisture (TWI) affect seedling health and survival was developed and seedlings were monitored 3 and 14 months after planting. Further monitoring will continue to examine how long term seedling success is affected by these variables. Future studies might address:

- How long term seedling survival is affected by the original ectomycorrhizal colonization status of seedlings at the time of planting; root sampling could be used to monitor the sustainability of colonization.
- How seedling height is influenced by ectomycorrhizal fungi and other variables.
- How seedling survival is affected by microsite type and position, and more specifically if seedlings planted with snags or those planted uphill of microsites will experience higher mortality.
- How seedling-level slope and aspect affect long term survival at the current study site; site-level slope and aspect as related to seedling health in the current planting could be compared to future plantings of varying slopes and aspects across the Eureka Burn.

- How long term seedling establishment is related to seedling-level TWI; results could be applied on a larger scale across the Eureka Burn.

The establishment of long term monitoring sites with well-documented planting information can be critical in the restoration of whitebark pine, and incorporation of modern GPS and GIS technology in this restoration allows for potentially impactful analyses that can influence future plantings. As shown in previous studies, the successes of plantings are not based on a single solution, but rather benefit from consideration of a plethora of variables that shape the natural ecology of whitebark pine. The collaboration of researchers and land managers in an effort to develop best planting practices and to capitalize on valuable seedling resources has the potential to influence the destiny of whitebark pine across the landscape of western North America.

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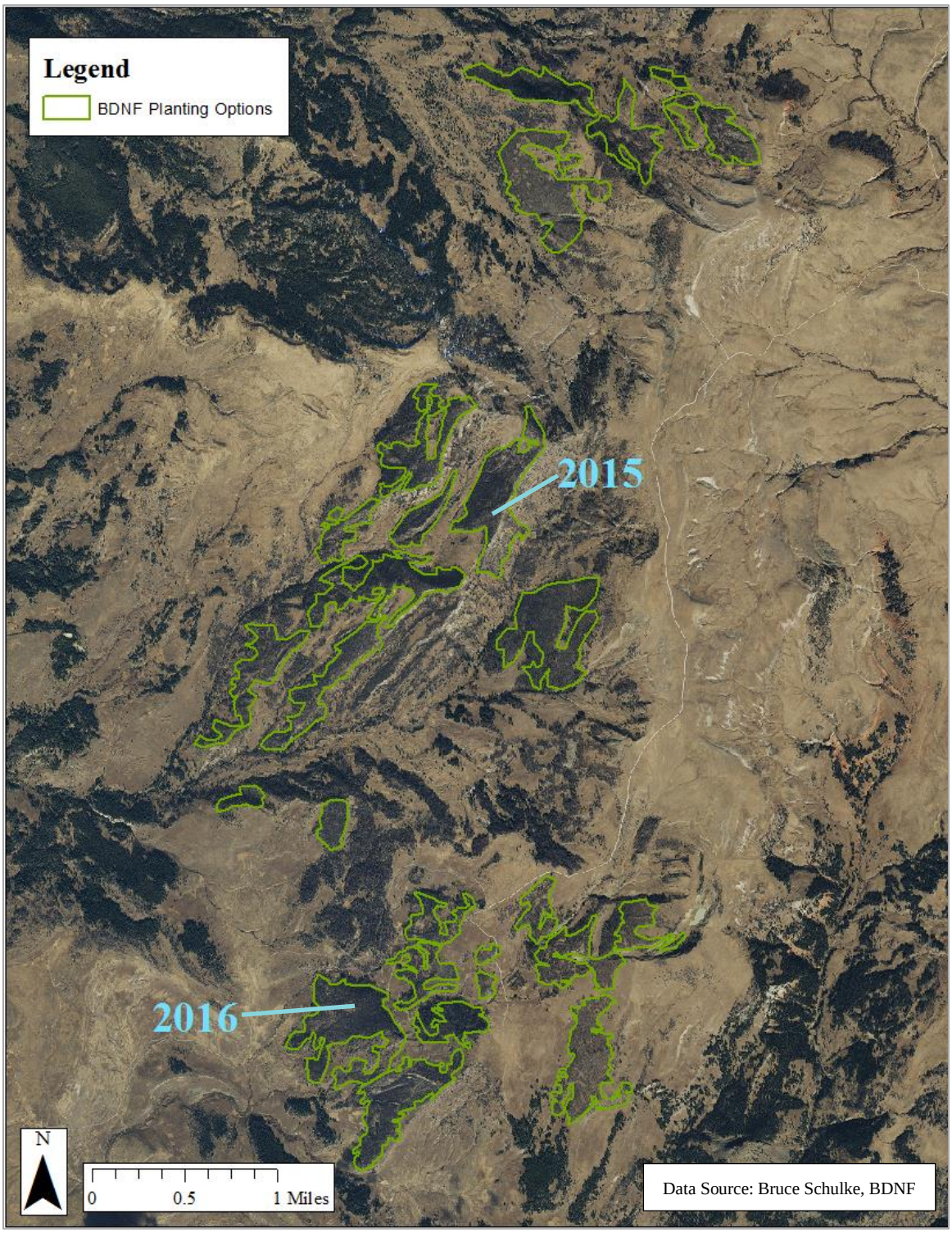
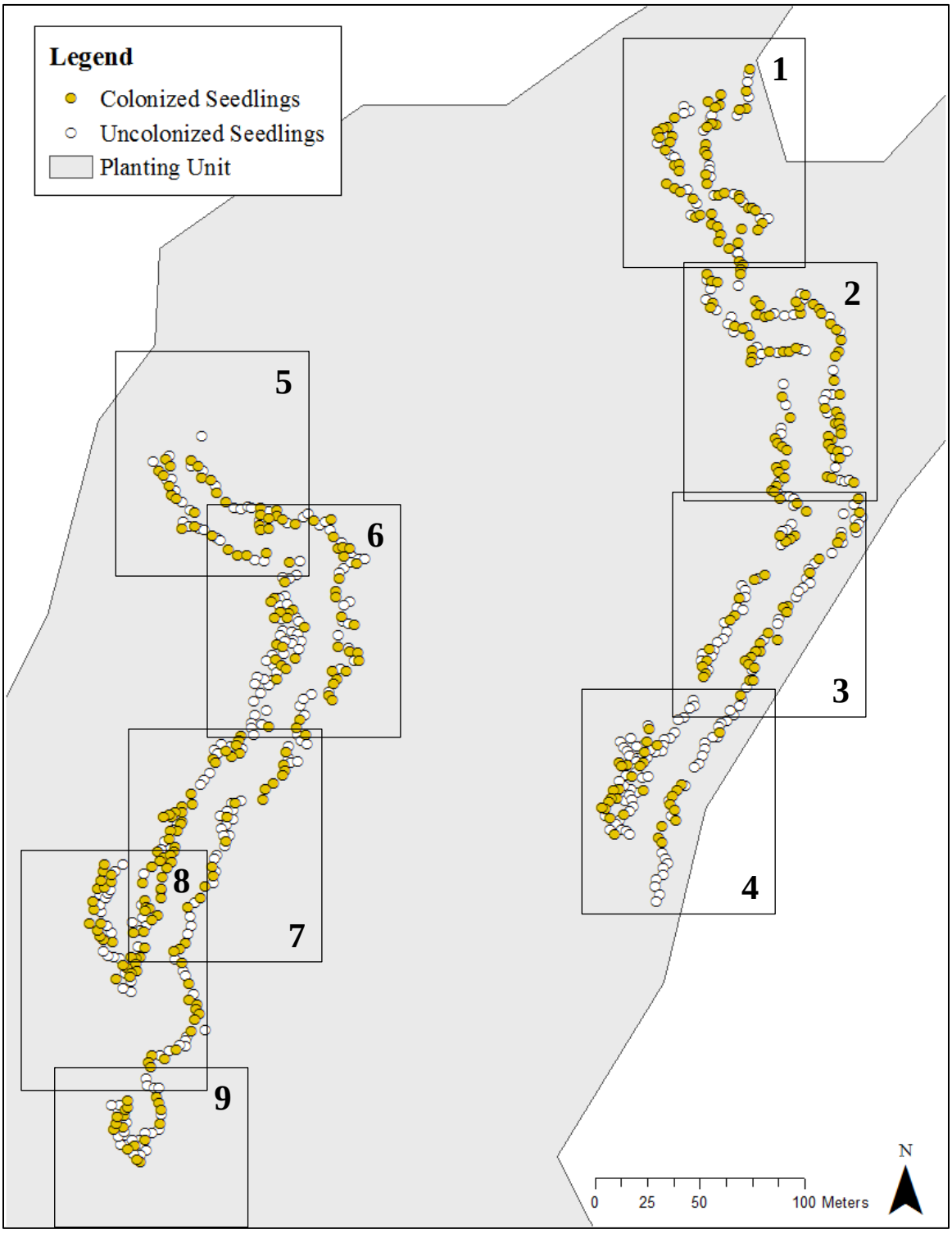
APPENDIX A

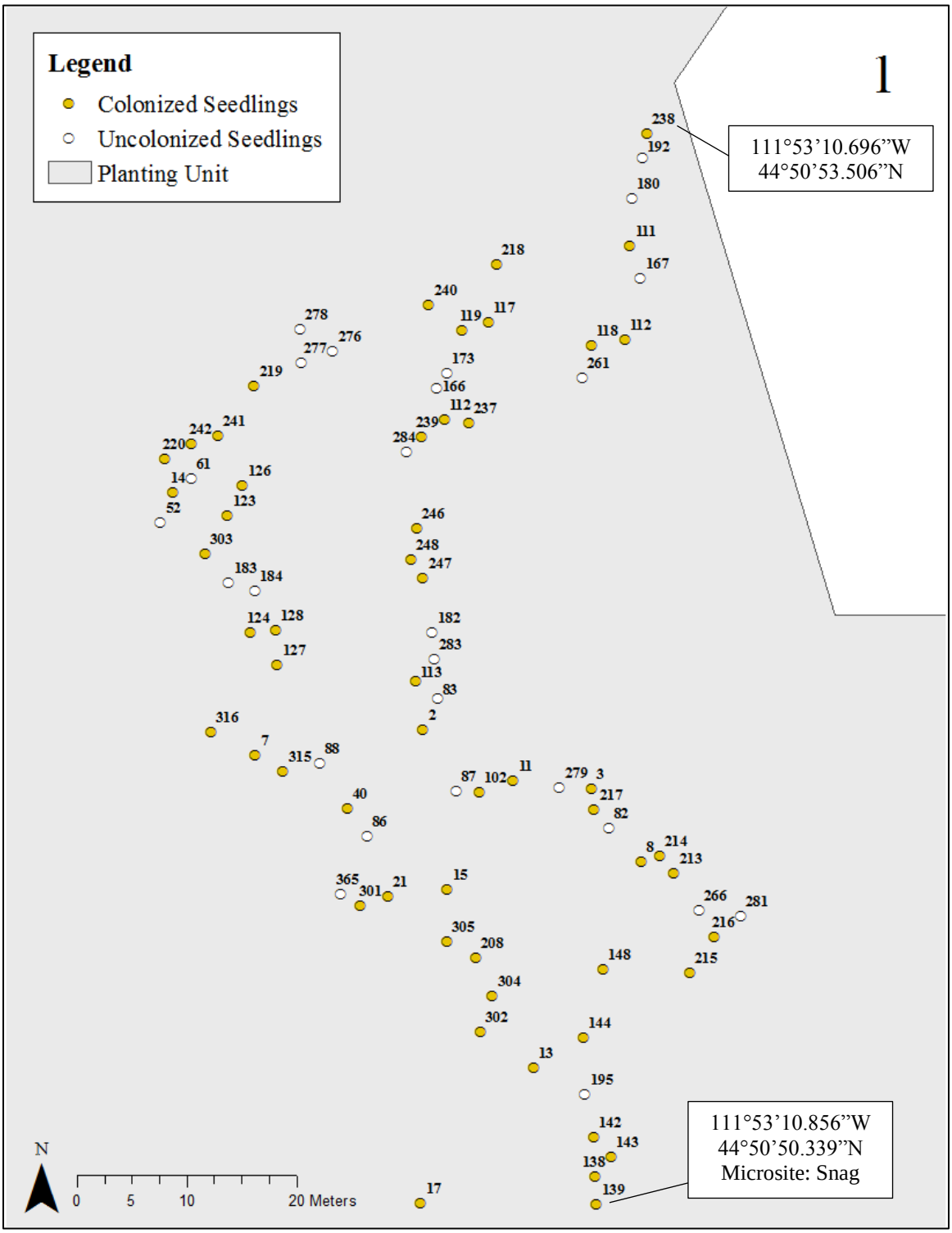
FIELD STUDY MAPS AND SEEDLING DATA

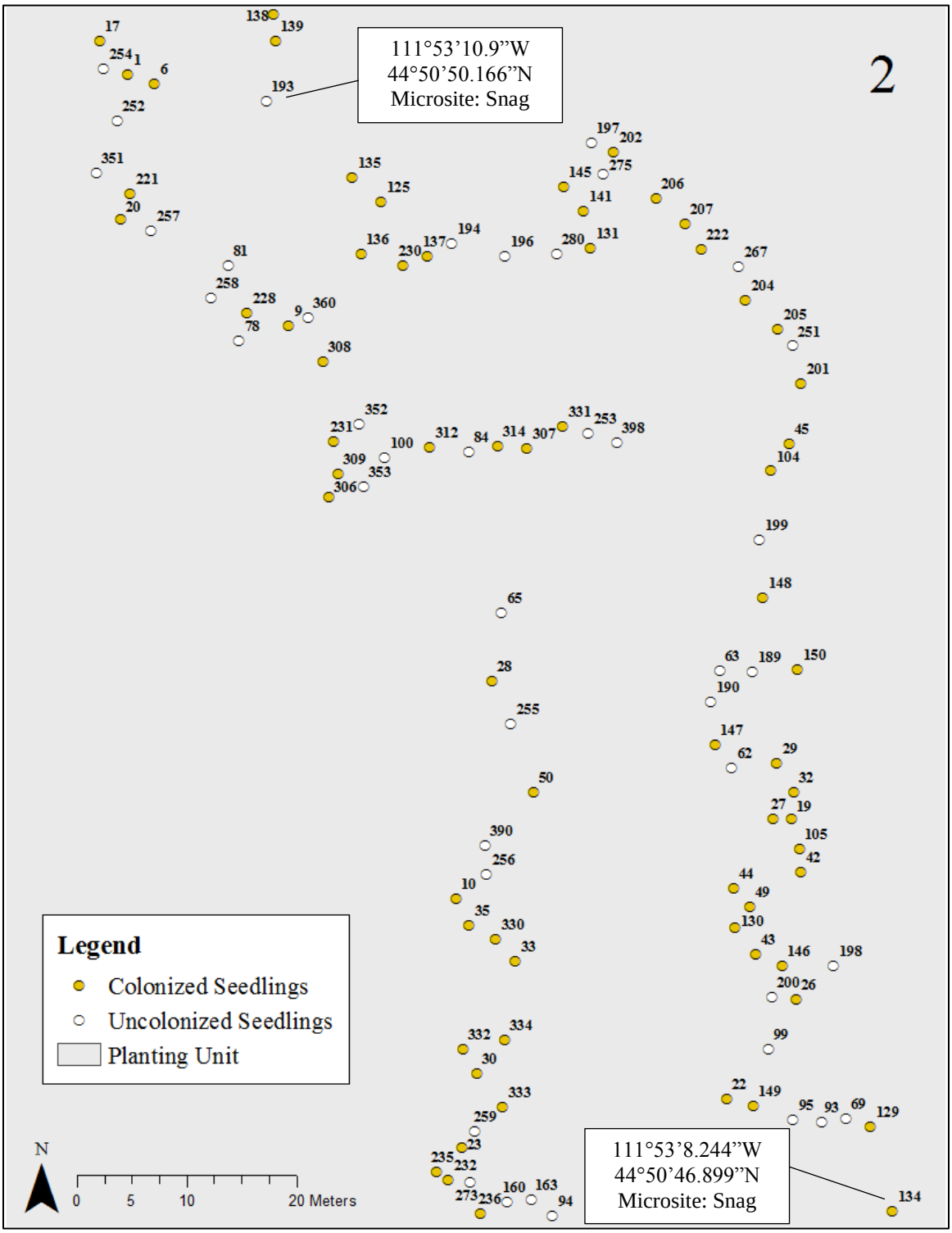
Beaverhead-Deerlodge National Forest planting options for the restoration of whitebark pine with the 2015 and 2016 planting sites labeled.

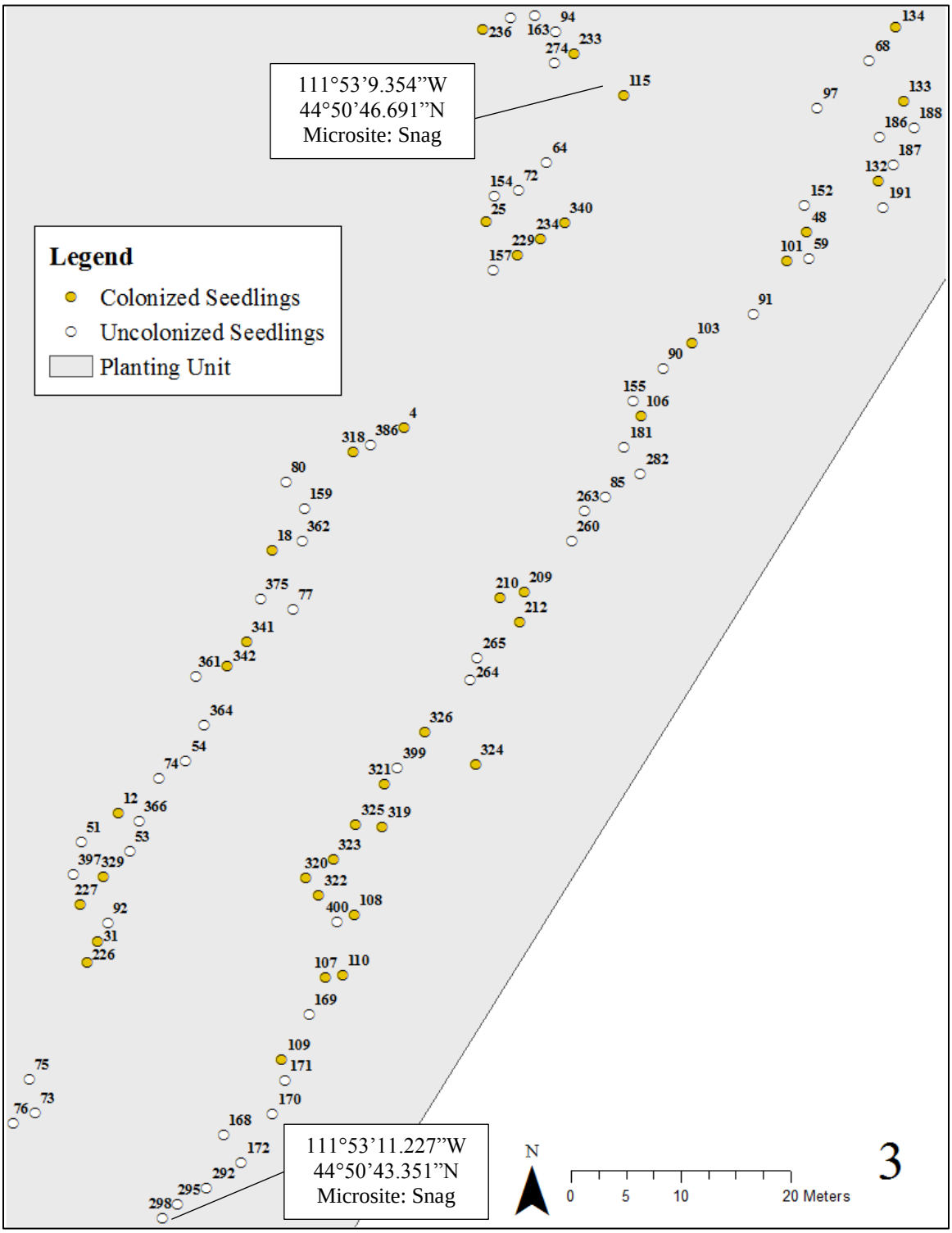


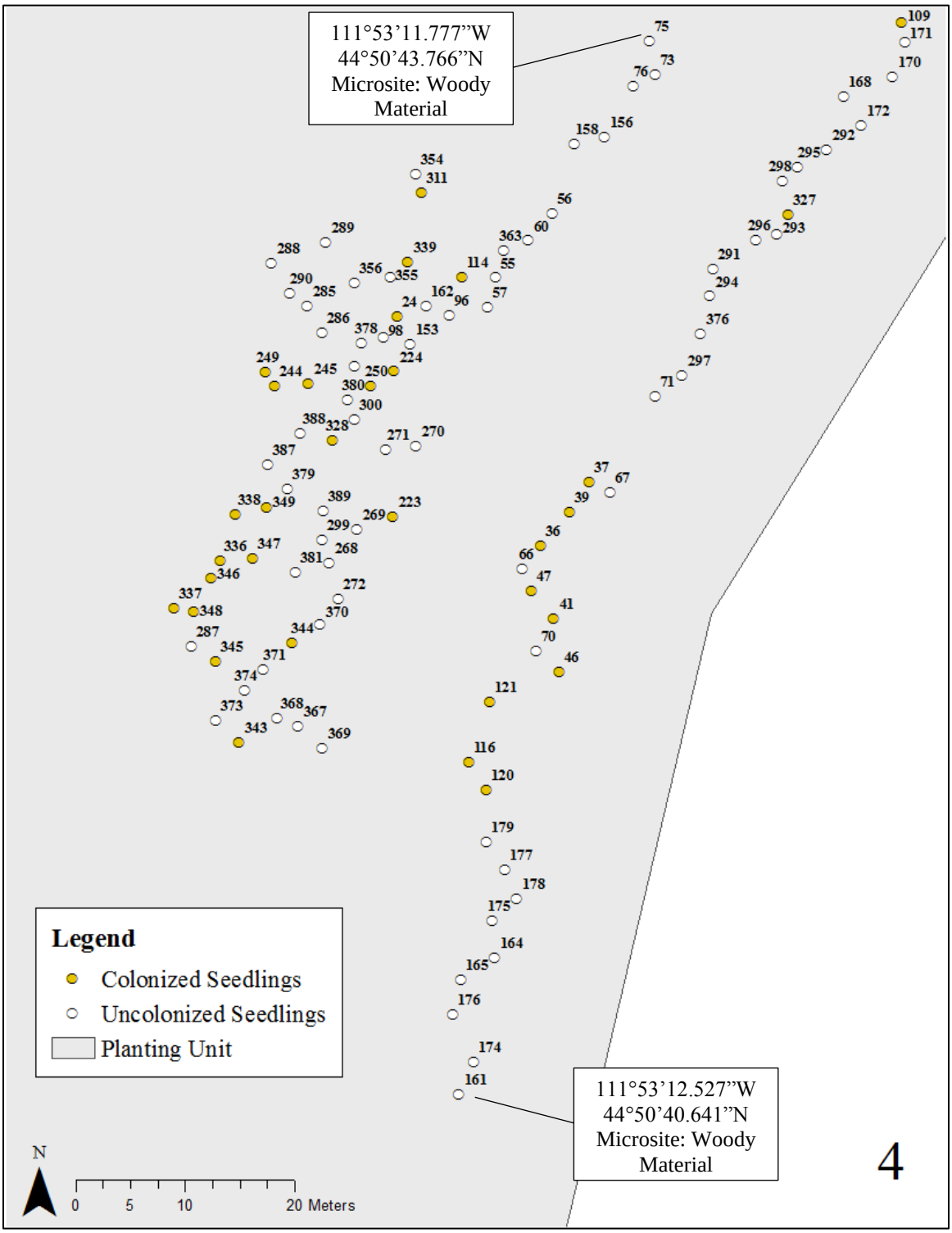
The 2015 study site with transects of tagged seedlings.

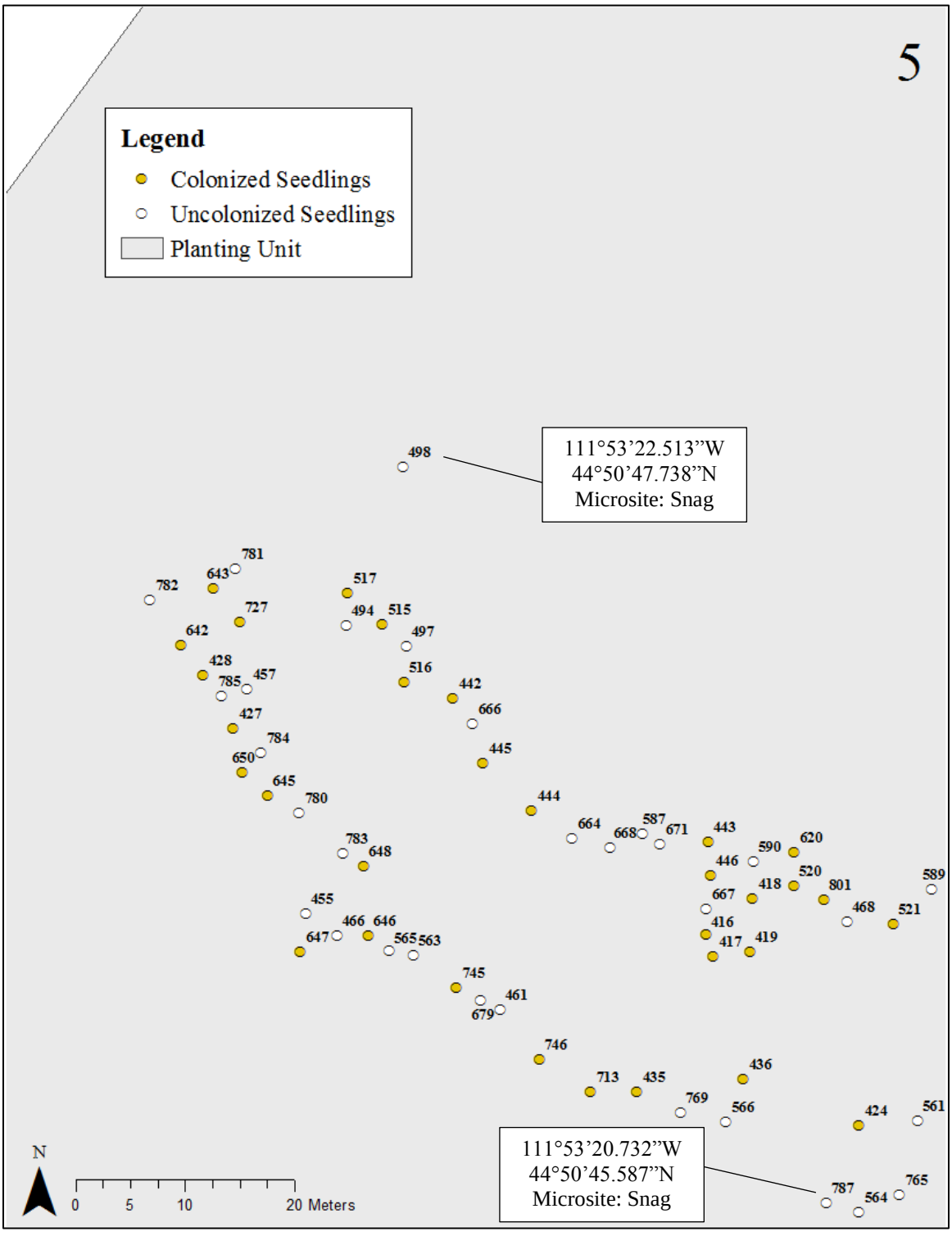



Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
1
111°53’10.696”W
44°50’53.506”N
111°53’10.856”W
44°50’50.339”N
Microsite: Snag
N
0
5
10
20 Meters


2
111°53’10.9”W
44°50’50.166”N
Microsite: Snag
111°53’8.244”W
44°50’46.899”N
Microsite: Snag
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
N
0
5
10
20 Meters


111°53’9.354”W
44°50’46.691”N
Microsite: Snag
111°53’11.227”W
44°50’43.351”N
Microsite: Snag
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
N
0
5
10
20 Meters
3


111°53’11.777”W
44°50’43.766”N
Microsite: Woody Material
111°53’12.527”W
44°50’40.641”N
Microsite: Woody Material
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
N
0
5
10
20 Meters
4


5
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
111°53’22.513”W
44°50’47.738”N
Microsite: Snag
111°53’20.732”W
44°50’45.587”N
Microsite: Snag
N
0 5 10 20 Meters
498
781
643
782
517
727
494
515
642
497
428
516
785
457
442
427
666
784
445
650
645
780
444
664
587
668
671
443
783
590
620
648
446
520
589
455
667
418
801
466
646
468
521
647
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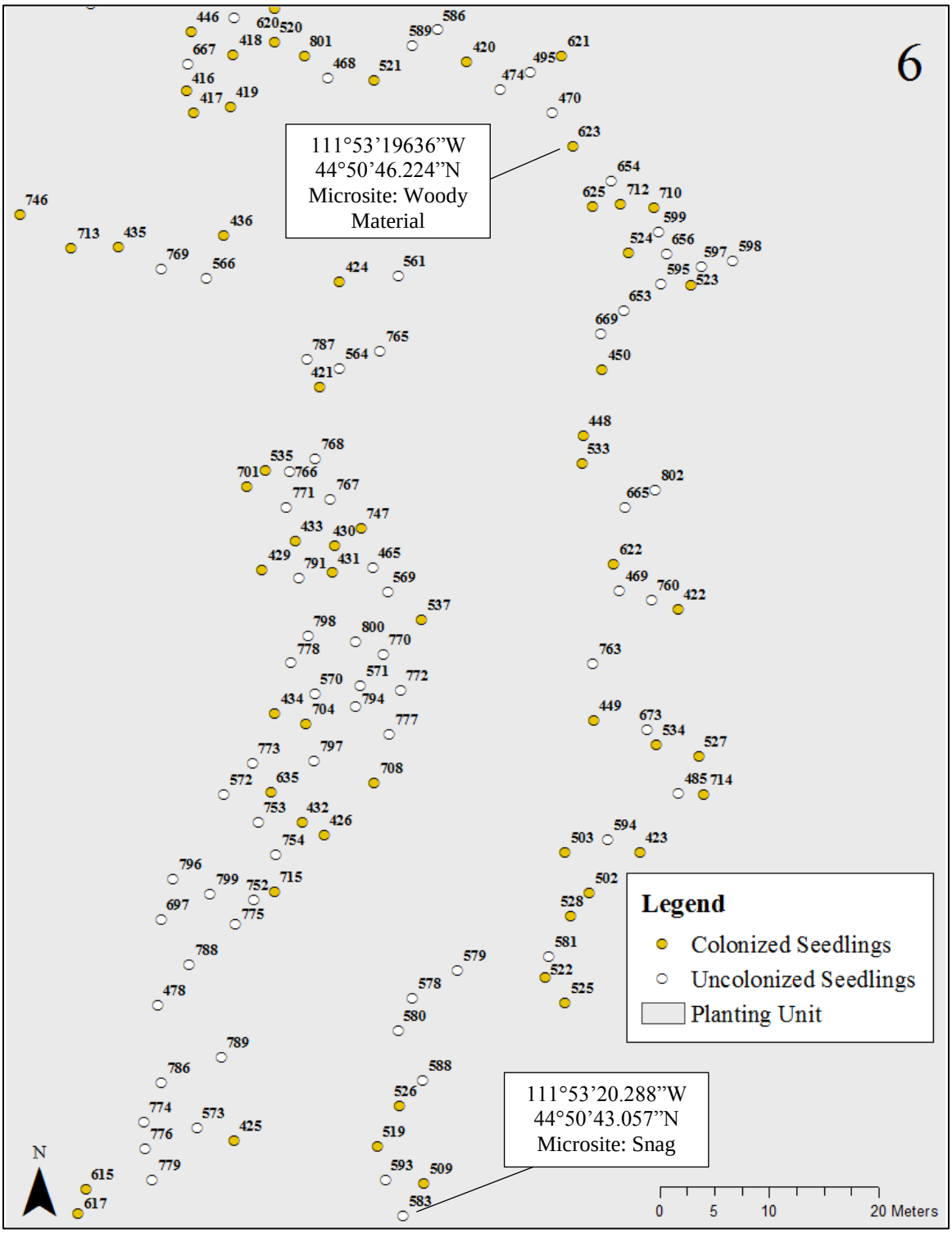
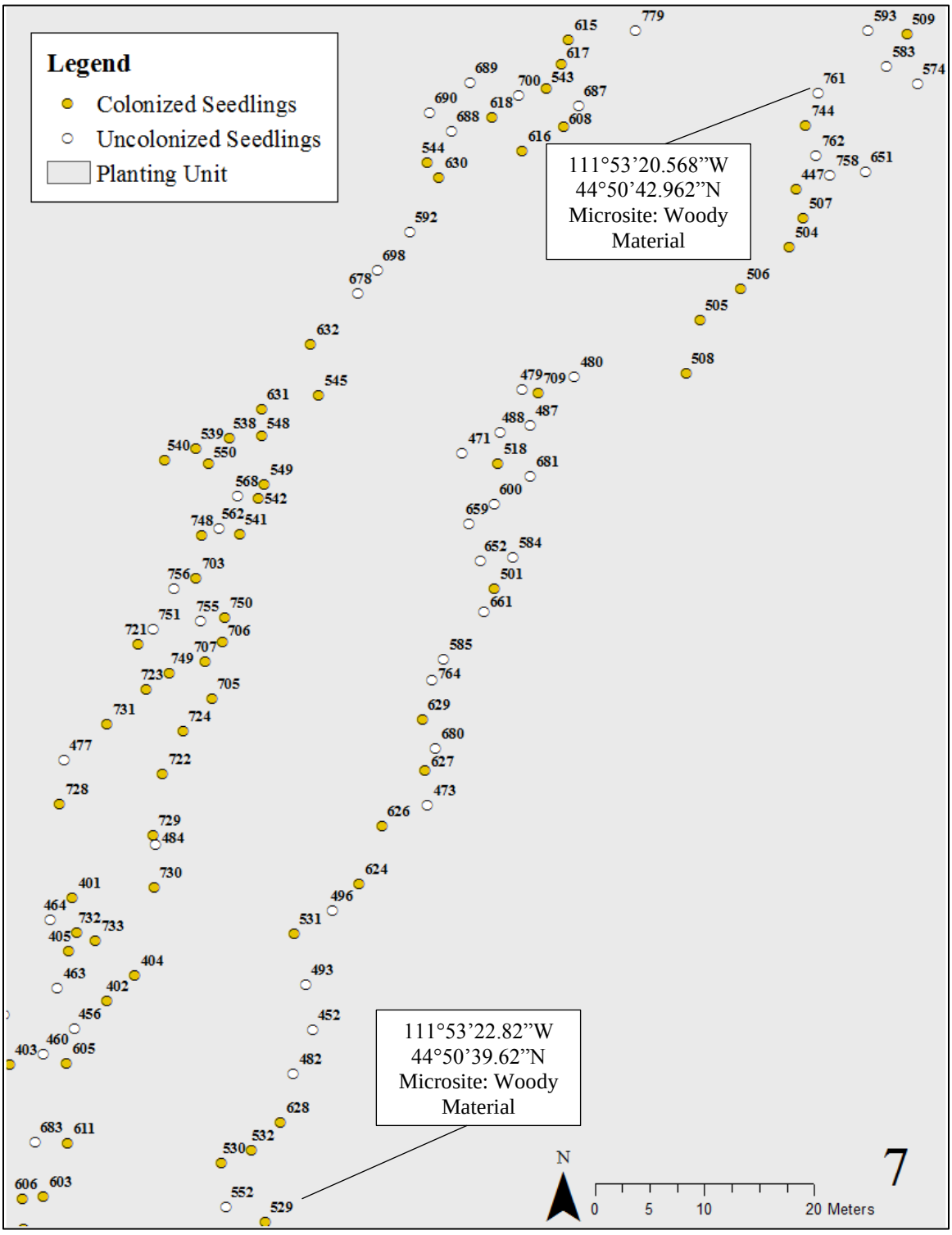
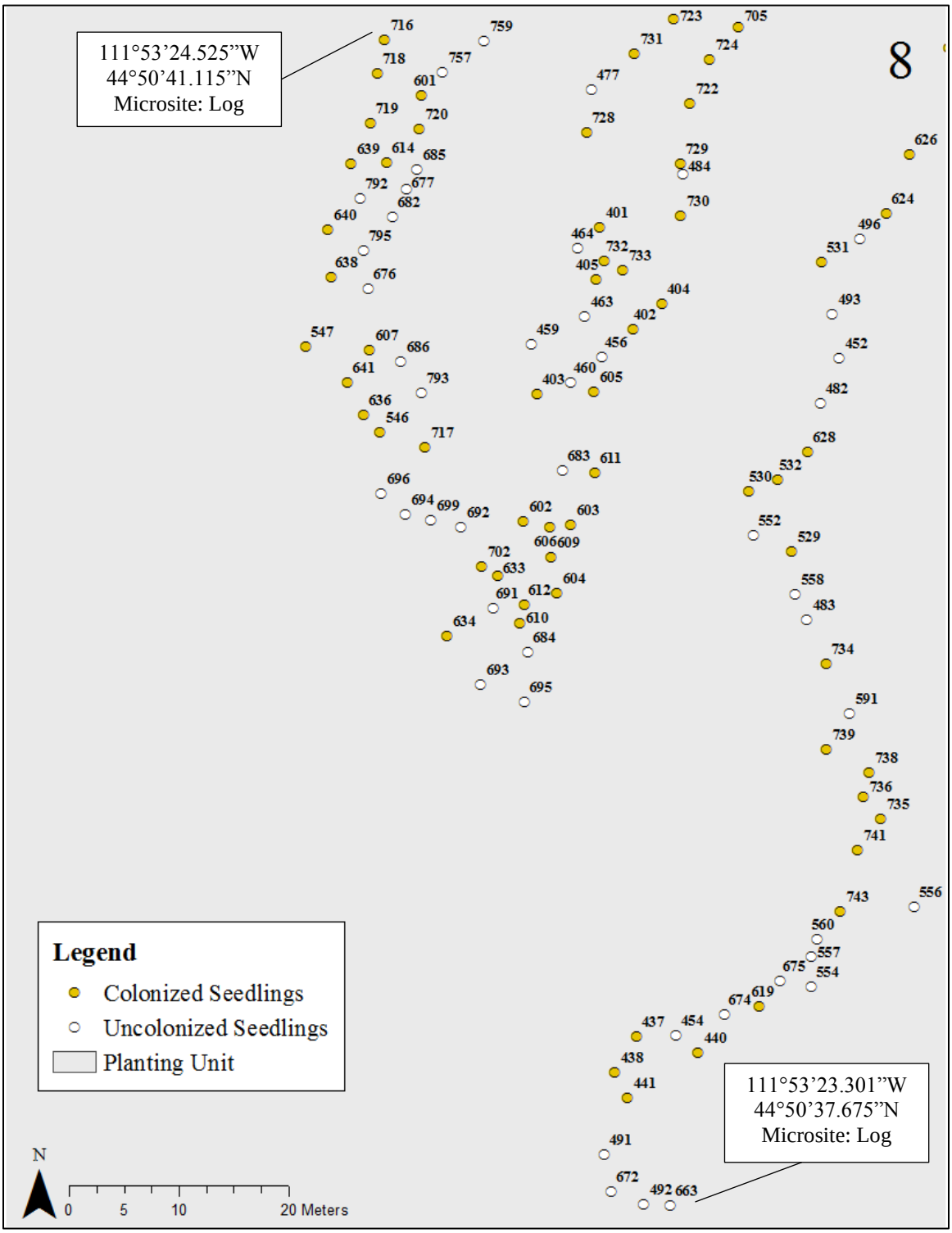
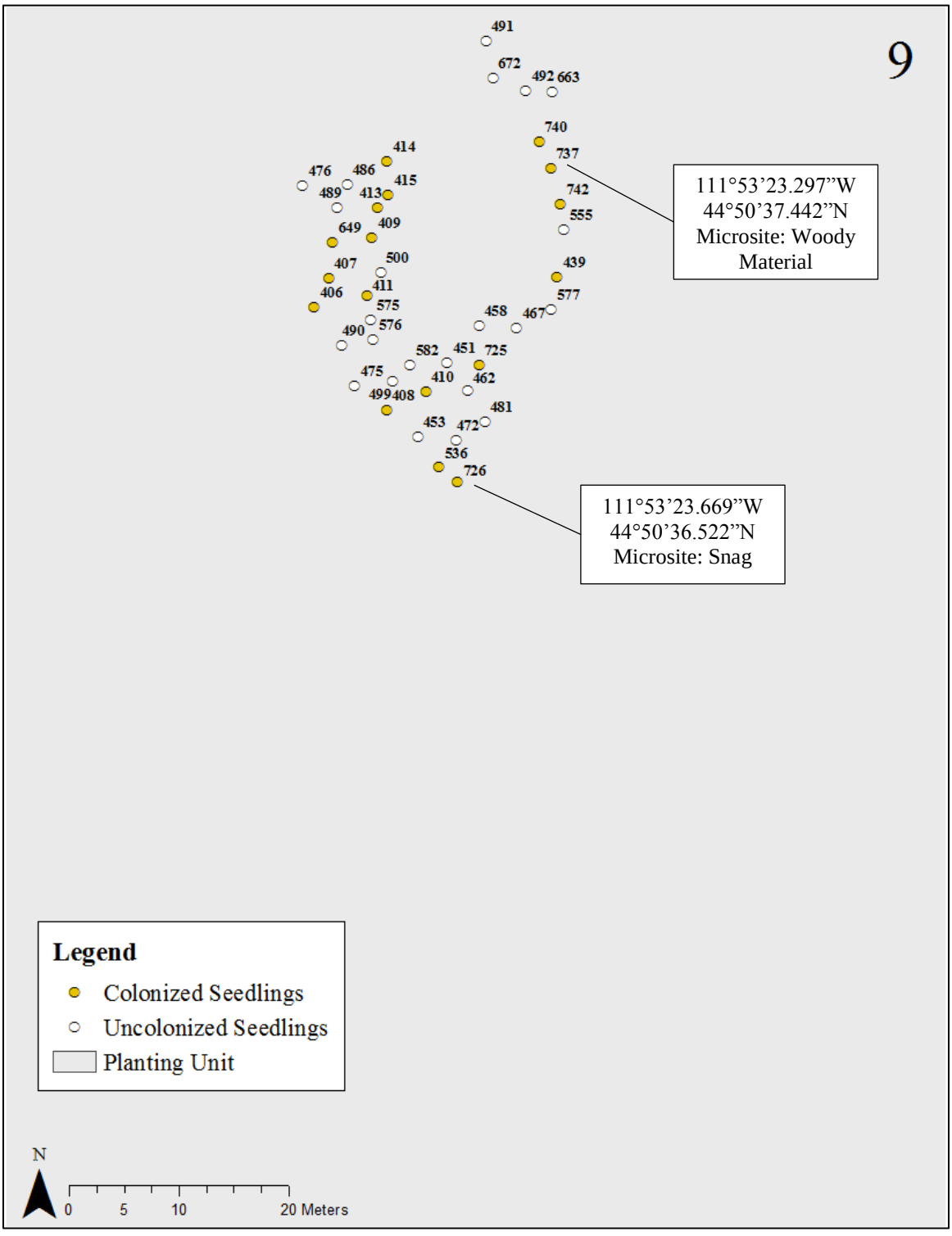
435
436
769
566
424
561
787
564
765


6
111°53’19636”W
44°50’46.224”N
Microsite: Woody Material
111°53’20.288”W
44°50’43.057”N
Microsite: Snag
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
N
0 5 10 20 Meters


Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
111°53’20.568”W
44°50’42.962”N
Microsite: Woody Material
111°53’22.82”W
44°50’39.62”N
Microsite: Woody Material
N
0 5 10 20 Meters
7


111°53’24.525”W
44°50’41.115”N
Microsite: Log
8
111°53’23.301”W
44°50’37.675”N
Microsite: Log
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
N
0
5
10
20 Meters


9
491
672
492
663
740
737
742
555
414
476
486
415
489
413
649
409
407
500
406
411
575
576
490
439
577
458
467
582
451
725
475
410
462
499
408
481
453
472
536
726
111°53’23.297”W
44°50’37.442”N
Microsite: Woody Material
111°53’23.669”W
44°50’36.522”N
Microsite: Snag
Legend
Colonized Seedlings
Uncolonized Seedlings
Planting Unit
N
0
5
10
20 Meters

Seedling Data

Tag Color:

Yellow = colonized by ECM fungi
White = not colonized by ECM fungi

Microsite Abbreviations:

L = log

W = woody material

SN = snag (standing dead)

SP = stump

E = exposed

Microsite Position Abbreviations:

S = side by side

D = downhill

U = uphill

Slope: clinometer determined

Health:

0 = dead

00 = dead due to obvious physical cause
1 = <25% of needles alive

2 = 25-50% of needles alive

3 = 50-75% of needles alive

4 = 75-100% of needles alive

Dates

Planted: June 23-25, 2015

2015 Health: September 26, 2015

2016 Health: September 10, 2016

ID	Tag	Microsite	Microsite Position	Slope (°)	Aspect	Initial Height (cm)	2015 Health	2016 Health	TWI
1	Yellow	L	S	10	W	5.4	4	4	9.55
2	Yellow	W	D	10	SW	5.3	4	4	8.65
3	Yellow	SN	D	30	S	7.9	4	4	9.21
4	Yellow	SN	U	10	W	5.1	4	4	6.82
5	Yellow	-	-	-	-	-	-	-	-
6	Yellow	L	S	10	W	5.4	4	4	9.55
7	Yellow	W	S	10	SW	9	4	4	8.20
8	Yellow	W	U	30	S	4.4	4	4	9.53
9	Yellow	SN	S	10	W	6.8	4	4	9.06
10	Yellow	L	S	5	SW	8	4	4	8.44
11	Yellow	SN	D	5	SW	7.4	4	4	9.11
12	Yellow	L	S	20	W	5.6	4	4	7.78
13	Yellow	SN	S	10	W	6.5	3	3	9.71
14	Yellow	W	S	15	SW	6	4	4	7.84
15	Yellow	L	S	35	S	7	4	4	9.18
16	Yellow	-	-	-	-	-	-	-	-
17	Yellow	SN	S	5	SW	7.7	4	4	9.59
18	Yellow	SN	S	10	W	6.3	4	4	7.12
19	Yellow	W	S	5	S	5.4	3	3	8.08
20	Yellow	L	D	5	NW	4.5	3	2	9.50
21	Yellow	SN	S	40	S	9	4	4	8.82
22	Yellow	W	S	5	W	7.1	4	4	7.59
23	Yellow	W	S	-	-	9.1	4	4	7.57
24	Yellow	L	U	20	W	4	4	4	7.75
25	Yellow	SN	U	5	W	4.4	4	4	6.88
26	Yellow	L	S	10	W	6.1	4	4	7.65
27	Yellow	L	D	5	SW	9	4	4	8.08
28	Yellow	SN	D	5	SW	8.1	4	4	8.65
29	Yellow	W	S	10	W	8.4	4	4	8.08
30	Yellow	SN	U	5	SW	7.7	4	4	7.94
31	Yellow	W	-	10	W	9.9	-	4	-
32	Yellow	W	S	5	W	5	4	4	8.08
33	Yellow	L	S	5	SW	7.4	4	4	8.10
34	Yellow	-	-	-	-	-	-	-	-

35	Yellow	L	S	5	SW	6.9	4	4	8.10
36	Yellow	W	S	10	W	7.5	4	4	6.96
37	Yellow	SN	S	5	W	5	4	4	7.17
38	Yellow	-	-	-	-	-	-	-	-
39	Yellow	SP	U	10	W	8.1	4	4	7.17
40	Yellow	SN	S	10	SW	5.5	4	4	8.54
41	Yellow	L	S	5	W	11	4	4	6.78
42	Yellow	W	D	5	S	6.4	4	4	7.85
43	Yellow	W	S	10	W	5.5	4	4	7.65
44	Yellow	SN	S	5	SW	8.6	4	4	8.01
45	Yellow	SN	S	5	W	5.5	4	4	8.23
46	Yellow	W	D	10	W	6.3	4	4	6.78
47	Yellow	W	S	10	W	8.1	4	4	6.96
48	Yellow	L	D	10	NW	6.2	4	4	6.51
49	Yellow	SN	D	5	SW	9.4	4	4	7.85
50	Yellow	W	U	5	SW	4	4	4	8.52
51	White	W	S	20	W	2.9	4	4	7.78
52	White	SN	S	10	SW	10.4	4	4	7.84
53	White	W	S	20	W	5	4	4	7.78
54	White	SN	U	20	W	4.2	4	4	7.55
55	White	SN	U	20	W	4.9	4	1	7.67
56	White	W	S	20	W	7	4	4	7.74
57	White	W	S	20	W	4	4	4	7.67
58	White	-	-	-	-	-	-	-	-
59	White	W	S	10	W	8.7	4	4	6.51
60	White	W	U	10	W	5.3	4	00	7.54
61	White	W	S	15	SW	10	4	4	7.84
62	White	L	D	10	W	4.4	3	4	8.24
63	White	SN	S	10	W	7.8	4	4	8.40
64	White	SN	S	10	W	5.9	4	4	7.10
65	White	W	S	5	SW	6.9	4	4	8.64
66	White	W	S	10	W	7.1	4	4	6.96
67	White	W	S	10	W	4	4	4	6.93
68	White	W	U	15	NW	4.5	4	4	6.90
69	White	W	S	10	NW	5.6	4	3	7.17
70	White	W	S	10	W	5.6	4	4	6.78
71	White	W	D	10	W	7.5	4	3	7.16
72	White	W	S	10	NW	4.7	4	4	7.10

73	White	W	U	10	W	3.8	4	4	7.72
74	White	SN	U	20	W	3.5	4	4	7.61
75	White	W	S	20	W	2.9	4	4	7.82
76	White	L	S	10	W	3.2	4	4	7.72
77	White	W	S	10	W	4.6	3	4	7.17
78	White	W	U	10	W	7.6	4	4	9.24
79	White	-	-	-	-	-	-	-	-
80	White	SN	S	10	W	6.8	4	4	7.07
81	White	W	D	10	S	5.5	4	4	9.24
82	White	-	S	30	S	4.9	4	3	9.21
83	White	SN	S	10	SW	4.5	3	4	8.65
84	White	W	S	10	SW	6.1	4	4	8.70
85	White	SN	S	5	W	3	4	4	6.37
86	White	L	S	10	SW	5	4	4	8.82
87	White	L	S	20	SW	9.4	4	4	8.95
88	White	L	D	10	SW	5	3	4	8.54
89	White	-	-	-	-	-	-	-	-
90	White	SN	S	5	W	3.1	4	3	6.35
91	White	SN	D	10	W	7	4	4	6.20
92	White	W	S	20	W	6.8	3	3	7.84
93	White	L	S	10	NW	3.2	4	4	7.20
94	White	SN	U	10	W	4	4	4	7.20
95	White	L	S	10	NW	3.3	4	4	7.20
96	White	W	S	20	W	4.8	3	4	7.67
97	White	W	-	15	NW	3.8	-	4	-
98	White	W	S	20	W	3.3	4	4	7.61
99	White	SN	D	10	W	9.5	3	4	7.55
100	White	SN	U	10	SW	5	3	4	8.81
101	Yellow	W	S	10	W	6	4	4	6.51
102	Yellow	SN	S	20	SW	8.5	4	4	8.95
103	Yellow	W	S	10	W	8	4	4	6.35
104	Yellow	W	S	5	S	5.1	4	4	8.23
105	Yellow	L	D	10	S	4.5	4	3	7.85
106	Yellow	SN	U	10	W	3.9	4	4	6.37
107	Yellow	SN	S	10	W	3.2	4	4	7.29
108	Yellow	W	S	10	W	8.2	4	4	7.29
109	Yellow	W	D	5	W	3.9	4	4	7.24
110	Yellow	W	S	10	W	6	4	4	7.29

111	Yellow	-	-	-	-	-	0	00	-
112	Yellow	W	D	10	SW	11	4	4	7.64
113	Yellow	SN	D	0	SW	4.6	4	4	8.65
114	Yellow	SN	S	10	W	8.1	3	4	7.67
115	Yellow	SN	U	10	W	7.7	4	4	7.08
116	Yellow	L	S	10	W	7.5	4	4	6.85
117	Yellow	W	D	0	SW	5.9	4	4	7.61
118	Yellow	W	D	10	SW	6.5	4	4	7.64
119	Yellow	-	-	-	-	-	-	4	-
120	Yellow	L	S	10	W	7.8	4	4	6.85
121	Yellow	W	S	5	W	6.1	4	4	7.03
122	Yellow	-	-	-	-	-	-	-	-
123	Yellow	SN	S	10	SW	4.6	4	4	7.81
124	Yellow	SN	D	5	SW	5	4	4	7.97
125	Yellow	W	S	5	W	7	4	4	9.12
126	Yellow	W	S	5	W	6.4	4	4	7.81
127	Yellow	W	D	10	SW	8.5	4	4	8.01
128	Yellow	SN	D	5	SW	6.9	4	4	7.97
129	Yellow	W	S	10	NW	6.7	4	4	7.17
130	Yellow	E	D	5	SW	9.1	4	4	7.78
131	Yellow	SN	S	10	NW	9	4	4	8.69
132	Yellow	W	S	5	NW	4.4	3	-	6.68
133	Yellow	SN	U	15	NW	4.5	4	4	6.90
134	Yellow	SN	S	20	NW	9	4	4	6.90
135	Yellow	SN	S	0	SW	6.5	4	4	9.38
136	Yellow	W	S	10	W	9	4	4	9.06
137	Yellow	L	U	5	NW	9.4	4	4	8.95
138	Yellow	L	S	10	SW	5.6	4	4	9.68
139	Yellow	SN	S	10	W	6.8	4	4	9.68
140	Yellow	SN	D	10	NW	7	4	4	-
141	Yellow	W	S	5	NW	11.1	4	4	8.75
142	Yellow	SN	S	5	W	10.7	4	4	9.68
143	Yellow	W	D	5	SW	8.2	4	4	9.68
144	Yellow	SN	D	10	SW	8.2	3	4	9.71
145	Yellow	W	S	10	NW	5.1	4	4	8.75
146	Yellow	SN	D	5	W	8.5	4	4	7.65
147	Yellow	L	D	10	W	6.3	4	4	8.24
148	Yellow	W	D	-	SW	9.8	4	4	8.16

149	Yellow	SN	S	5	W	6.9	4	4	7.55
150	Yellow	SN	D	5	W	9.2	4	4	8.21
151	White	-	-	-	-	-	-	-	-
152	White	L	D	10	NW	3.9	3	4	6.75
153	White	W	U	20	W	7.2	4	4	7.61
154	White	SN	U	10	W	7.3	4	4	7.10
155	White	W	S	10	W	4.7	4	4	6.47
156	White	SN	U	10	W	3	4	4	7.72
157	White	L	S	10	W	5.9	4	4	6.88
158	White	SN	S	10	W	3.7	4	4	7.74
159	White	W	S	10	W	6.4	4	4	7.12
160	White	W	S	10	W	4.3	4	4	7.57
161	White	W	S	10	W	5.4	4	4	6.48
162	White	SN	S	10	W	2.5	4	4	7.67
163	White	-	S	10	W	5.9	4	4	7.57
164	White	SN	U	10	W	10.4	4	2	6.50
165	White	SN	U	10	W	6.1	4	4	6.50
166	White	W	U	10	SW	3.8	4	4	7.72
167	White	SN	S	5	SW	4.5	4	4	7.64
168	White	L	S	10	W	3.7	4	4	7.38
169	White	W	U	10	W	4.8	3	4	7.24
170	White	L	S	10	W	4.9	4	4	7.05
171	White	W	D	5	W	4.2	4	4	7.05
172	White	L	S	10	SW	3.7	4	4	7.38
173	White	W	U	20	SW	6.5	3	1	7.72
174	White	SN	S	10	W	3.8	4	4	6.48
175	White	SN	U	10	W	7.5	4	4	6.50
176	White	SN	S	10	W	8.4	3	2	6.48
177	White	L	D	10	W	4.8	4	4	6.65
178	White	L	D	10	W	7.1	3	4	6.48
179	White	SN	S	5	W	6	4	4	6.65
180	White	SN	-	5	NW	5	-	4	-
181	White	W	S	5	W	4.8	4	4	6.37
182	White	SN	S	5	SW	6.1	4	4	8.39
183	White	SN	S	10	SW	3.6	4	4	7.97
184	White	SN	S	-	-	4.8	4	4	7.97
185	White	-	-	-	-	-	-	-	-
186	White	SN	S	10	NW	6.9	4	4	6.68

187	White	W	S	5	NW	3.7	4	4	6.68
188	White	W	S	5	NW	4	4	4	6.68
189	White	SN	D	10	W	4.5	4	4	8.21
190	White	L	D	10	W	7.4	4	4	8.40
191	White	W	S	5	NW	6	4	4	6.68
192	White	W	D	10	NW	5.1	4	4	7.44
193	White	SN	S	10	W	7	4	4	9.64
194	White	L	U	5	NW	5.3	4	4	9.12
195	White	SN	S	5	W	4.8	4	3	9.71
196	White	W	U	5	NW	6	4	4	8.82
197	White	W	S	10	S	5.5	4	4	9.01
198	White	L	S	10	W	4.6	4	4	7.65
199	White	SN	D	5	S	8.9	4	4	8.16
200	White	L	S	10	W	7	4	3	7.65
201	Yellow	SN	S	5	NW	6	4	4	8.31
202	Yellow	W	D	5	SW	9.8	4	4	9.01
203	Yellow	SP	S	5	NW	6.7	4	4	-
204	Yellow	W	D	20	NW	5.6	4	4	8.56
205	Yellow	SP	U	15	NW	7	4	4	8.42
206	Yellow	SN	S	10	NW	10.1	4	4	8.64
207	Yellow	L	S	10	NW	7.3	4	4	8.64
208	Yellow	L	U	35	N	2	4	4	9.42
209	Yellow	W	U	5	W	8	4	4	6.59
210	Yellow	SN	S	5	W	6	4	4	6.59
211	Yellow	-	-	-	-	-	-	-	-
212	Yellow	W	S	5	W	9.6	4	4	6.51
213	Yellow	W	S	40	S	6.6	4	3	9.53
214	Yellow	W	S	45	S	6	4	4	9.53
215	Yellow	SN	S	20	N	5.2	4	4	9.81
216	Yellow	SN	U	40	N	7.6	4	4	9.81
217	Yellow	W	S	40	S	5.3	4	4	9.21
218	Yellow	SN	S	0	SW	5.5	4	2	7.57
219	Yellow	SN	S	10	W	12.7	4	4	7.84
220	Yellow	SN	S	10	W	4	4	4	7.84
221	Yellow	SN	U	10	NW	6.1	4	4	9.50
222	Yellow	L	S	10	NW	8	4	4	8.56
223	Yellow	SN	S	20	W	6.6	4	4	7.49
224	Yellow	L	S	20	W	4	4	1	7.61

225	Yellow	SN	U	20	W	5.5	4	4	-
226	Yellow	W	S	20	W	5.6	4	4	7.84
227	Yellow	L	S	20	W	6.5	4	4	7.84
228	Yellow	L	S	10	W	9.5	4	4	9.24
229	Yellow	L	S	10	W	9.1	4	4	6.88
230	Yellow	L	U	5	W	6.7	4	4	8.95
231	Yellow	L	S	10	W	8	4	4	8.88
232	Yellow	L	S	10	W	7.2	4	4	7.79
233	Yellow	SN	U	10	W	6.3	4	4	7.08
234	Yellow	L	S	10	W	8.9	4	4	6.88
235	Yellow	L	S	10	W	7.2	4	4	7.79
236	Yellow	W	S	5	W	8.7	4	4	7.20
237	Yellow	W	D	0	SW	6.5	4	4	7.72
238	Yellow	-	S	-	-	-	4	-	7.44
239	Yellow	W	D	10	SW	6.2	4	4	7.72
240	Yellow	W	U	20	SW	6.3	4	4	7.61
241	Yellow	L	S	10	SW	8	3	00	7.84
242	Yellow	L	S	10	W	6.9	4	4	8.03
243	Yellow	W	S	10	SW	6.4	4	4	-
244	Yellow	SN	S	20	W	8.5	4	4	7.76
245	Yellow	SN	U	20	W	8.5	4	4	7.76
246	Yellow	SN	S	5	SW	6.2	3	3	8.01
247	Yellow	SN	S	5	SW	4.9	4	4	8.39
248	Yellow	W	S	5	SW	6.3	3	4	8.39
249	Yellow	SN	S	20	W	7.7	4	4	7.76
250	Yellow	SN	U	20	W	11	4	4	7.61
251	White	W	S	10	NW	5.1	4	3	8.31
252	White	SN	S	10	NW	7	4	4	9.55
253	White	SN	D	10	SW	6.9	4	4	8.62
254	White	W	S	5	W	7	4	4	9.55
255	White	SN	S	5	SW	4.9	4	4	8.65
256	White	L	S	5	SW	4.4	4	4	8.30
257	White	W	S	5	NW	3.8	4	4	9.50
258	White	W	U	5	SE	4.8	4	4	9.24
259	White	W	S	5	W	2.4	3	00	7.57
260	White	L	S	5	W	4	4	4	6.31
261	White	SN	S	10	W	6.1	4	4	7.81
262	White	-	-	-	-	-	-	-	-

263	White	W	U	5	W	3	4	4	6.31
264	White	W	S	5	W	4	4	4	6.51
265	White	W	U	5	W	5	4	4	6.51
266	White	L	U	30	N	6	4	3	9.75
267	White	SN	S	10	NW	10.5	4	4	8.56
268	White	L	U	20	W	5.3	4	4	7.34
269	White	SN	S	20	W	3.4	3	4	7.34
270	White	SN	S	20	W	6.1	3	2	7.37
271	White	W	S	20	W	5	4	4	7.49
272	White	SN	U	10	SW	2	4	4	7.34
273	White	L	S	10	W	6.4	4	4	7.57
274	White	W	U	10	W	3.1	4	4	7.20
275	White	L	D	5	NW	4.9	4	4	8.75
276	White	L	S	-	-	3.9	4	4	7.78
277	White	L	D	20	SW	4.9	4	4	7.72
278	White	W	S	10	SW	7	4	4	8.01
279	White	L	D	20	SW	3	3	3	9.11
280	White	SN	S	10	NW	5.5	4	4	8.69
281	White	SN	S	30	N	6	4	4	9.75
282	White	SP	D	0	W	5.9	4	4	6.37
283	White	SN	S	10	SW	9	3	4	8.65
284	White	W	S	10	SW	5.6	4	4	8.01
285	White	L	S	-	-	4.5	3	3	7.89
286	White	L	S	20	W	5.9	4	4	7.61
287	White	SN	U	20	W	8	4	4	7.56
288	White	L	S	20	W	3.5	4	2	7.89
289	White	W	S	20	W	4.9	4	4	7.75
290	White	L	S	20	W	2.6	3	2	7.89
291	White	SN	U	10	W	3	4	4	7.11
292	White	W	U	5	W	5	3	4	7.11
293	White	W	S	5	W	3.7	4	4	7.11
294	White	W	S	10	W	3.5	4	4	7.11
295	White	SN	U	10	W	4.8	4	4	7.11
296	White	SN	U	10	W	3.7	4	4	7.11
297	White	L	S	5	W	2.3	4	3	7.16
298	White	SN	S	5	W	2.8	4	4	7.46
299	White	L	U	20	W	4.6	4	4	7.34
300	White	W	S	20	W	4.4	4	4	7.61

301	Yellow	SN	S	40	S	4.5	4	3	8.82
302	Yellow	SN	S	10	W	6.5	4	4	9.54
303	Yellow	W	S	10	SW	8.5	4	4	7.97
304	Yellow	SN	U	10	NW	6.9	4	4	9.42
305	Yellow	W	D	40	N	7.3	4	4	9.42
306	Yellow	SN	S	10	SW	3.9	4	4	8.88
307	Yellow	L	S	10	SW	9.1	3	4	8.70
308	Yellow	SN	S	5	W	9.1	4	4	8.95
309	Yellow	L	D	10	SW	8	4	4	8.88
310	Yellow	-	-	-	-	-	-	-	-
311	Yellow	L	D	20	W	4.6	4	4	7.83
312	Yellow	SN	U	10	SW	4.6	4	4	8.81
313	Yellow	-	-	-	-	-	-	-	-
314	Yellow	L	S	10	SW	5.8	4	4	8.70
315	Yellow	W	S	10	SW	7	4	4	8.20
316	Yellow	SN	D	5	SW	8.1	4	4	8.01
317	Yellow	-	-	-	-	-	-	-	-
318	Yellow	W	S	10	W	6.6	4	4	7.07
319	Yellow	W	S	5	W	3	4	4	7.08
320	Yellow	W	S	10	W	4.9	4	4	7.43
321	Yellow	SN	U	5	W	5.5	4	4	6.96
322	Yellow	E	NA	10	W	5	4	4	7.29
323	Yellow	SN	U	10	W	5	4	4	7.43
324	Yellow	W	S	5	W	8	4	4	6.64
325	Yellow	SN	S	10	W	4	4	4	7.43
326	Yellow	SN	U	5	W	6	4	4	6.96
327	Yellow	W	U	10	W	3	4	4	7.46
328	Yellow	L	S	20	W	6.1	4	4	7.49
329	Yellow	SN	S	20	W	7.1	4	4	7.78
330	Yellow	L	S	5	SW	6.7	4	4	8.10
331	Yellow	W	D	10	SW	3.4	4	4	8.62
332	Yellow	W	D	5	SW	8.4	4	4	7.94
333	Yellow	L	U	10	W	5.6	4	4	7.94
334	Yellow	SN	S	5	SW	6	4	4	7.94
335	Yellow	-	-	-	-	-	-	-	-
336	Yellow	SN	S	20	W	6.1	4	4	7.46
337	Yellow	SN	U	20	W	8.9	4	4	7.66
338	Yellow	SN	S	20	W	5.5	4	4	7.63

339	Yellow	W	D	20	W	9.1	4	4	7.75
340	Yellow	L	S	10	W	7.8	4	4	6.72
341	Yellow	SN	U	10	W	6.3	4	4	7.42
342	Yellow	E	U	20	W	5.2	4	4	7.42
343	Yellow	SN	S	20	W	8.1	4	4	7.24
344	Yellow	SN	S	20	W	6.1	4	4	7.35
345	Yellow	SN	U	20	W	8.8	4	4	7.56
346	Yellow	SN	U	20	W	6.4	4	4	7.66
347	Yellow	W	D	20	W	6.3	4	4	7.46
348	Yellow	W	S	20	W	7.1	4	4	7.66
349	Yellow	SN	S	20	W	8.5	4	4	7.63
350	Yellow	-	-	-	-	-	-	-	-
351	White	L	S	20	NW	6.5	4	4	9.50
352	White	L	S	5	SW	8.1	4	4	8.95
353	White	SN	S	10	SW	4.9	0	0	8.88
354	White	L	D	20	W	7.5	4	4	7.83
355	White	SN	S	20	W	8.9	4	4	7.75
356	White	SN	U	20	W	12.1	4	4	7.75
357	White	-	-	-	-	-	-	-	-
358	White	-	-	-	-	-	-	-	-
359	White	-	-	-	-	-	-	-	-
360	White	SN	S	10	W	5.4	4	4	9.06
361	White	SN	S	20	W	6.7	4	4	7.42
362	White	SN	S	-	-	3.7	4	4	7.12
363	White	W	S	20	W	5.2	4	4	7.67
364	White	SN	U	20	W	3.5	4	4	7.55
365	White	W	S	15	S	1.5	4	4	8.82
366	White	L	S	20	W	3.6	4	4	7.78
367	White	W	S	20	W	6	4	4	7.24
368	White	SN	S	20	W	5.7	4	4	7.24
369	White	W	S	20	W	4	2	00	7.07
370	White	SN	S	-	-	7	4	4	7.21
371	White	W	S	20	W	5.7	3	4	7.35
372	White	SN	-	20	W	4.7	4	4	-
373	White	W	S	20	W	3.6	4	4	7.44
374	White	SN	S	20	W	2.7	3	3	7.35
375	White	W	S	10	W	7.4	4	4	7.31
376	White	SN	S	10	W	3.1	4	4	6.88

377	White	SN	U	20	W	7.3	4	4	7.61
378	White	L	S	20	W	5.1	4	1	7.61
379	White	W	S	20	W	3	3	2	7.63
380	White	SN	U	20	W	6.1	3	4	7.61
381	White	L	U	20	W	1.8	3	3	7.46
382	White	-	-	20	W	-	-	-	-
383	White	-	-	-	-	-	-	-	-
384	White	-	-	-	-	-	-	-	-
385	White	-	-	-	-	-	-	-	-
386	White	W	S	10	W	3.2	4	4	6.82
387	White	W	D	-	-	6.1	4	3	7.63
388	White	W	S	20	W	6.6	4	4	7.63
389	White	SN	S	20	W	4.1	4	4	7.49
390	White	W	D	5	SW	7.8	4	4	8.30
391	White	-	-	-	-	-	-	-	-
392	White	-	-	-	-	-	-	-	-
393	White	-	-	-	-	-	-	-	-
394	White	-	-	-	-	-	-	-	-
395	White	-	-	-	-	-	-	-	-
396	White	-	-	-	-	-	-	-	-
397	White	SP	S	20	W	5.7	4	4	7.69
398	White	SP	U	10	SW	3.9	4	4	8.57
399	White	SN	S	5	W	8	4	4	6.96
400	White	W	S	10	W	6.3	4	4	7.29
401	Yellow	W	S	10	W	7.1	4	4	9.26
402	Yellow	SN	D	10	W	5	4	4	9.09
403	Yellow	L	S	10	W	3.4	4	4	9.22
404	Yellow	L	S	10	W	9.4	4	3	9.09
405	Yellow	W	S	10	W	5	4	4	9.24
406	Yellow	W	S	5	W	8.2	4	3	8.91
407	Yellow	L	D	5	W	7.5	4	4	8.91
408	Yellow	L	D	10	W	7.5	4	3	8.45
409	Yellow	W	D	5	W	3.6	4	4	9.18
410	Yellow	W	D	10	W	7.2	4	3	8.61
411	Yellow	SN	S	5	W	7.1	4	4	8.91
412	Yellow	-	-	-	-	-	-	-	-
413	Yellow	SN	S	5	W	11	4	4	9.18
414	Yellow	SN	S	5	W	5	4	4	9.42

415	Yellow	SN	S	5	W	4.8	4	4	9.18
416	Yellow	L	D	10	W	7.4	4	4	8.39
417	Yellow	L	D	10	W	6	4	4	8.39
418	Yellow	W	S	10	W	4.8	4	3	8.82
419	Yellow	SN	D	10	W	6.9	4	4	8.82
420	Yellow	SN	D	5	W	4.1	4	4	9.27
421	Yellow	W	D	10	NW	5	4	4	9.60
422	Yellow	L	U	20	W	8	4	4	8.20
423	Yellow	SN	U	10	W	6.1	4	4	9.14
424	Yellow	L	D	10	NW	9	4	3	9.50
425	Yellow	SN	D	10	W	4.1	4	4	9.13
426	Yellow	W	U	10	W	5.7	4	4	9.40
427	Yellow	SN	D	10	W	9	4	4	8.33
428	Yellow	L	D	10	W	6.4	4	4	8.56
429	Yellow	L	S	10	W	7.5	4	4	9.61
430	Yellow	L	S	10	W	4.3	4	3	9.32
431	Yellow	L	S	10	W	7.9	4	4	9.32
432	Yellow	W	D	10	W	6	4	4	9.40
433	Yellow	SN	D	10	W	3.2	4	4	9.32
434	Yellow	L	D	10	W	6	4	4	9.47
435	Yellow	W	S	20	SW	8.5	4	4	9.63
436	Yellow	W	D	10	W	9	4	4	9.63
437	Yellow	L	S	10	W	4.5	4	4	9.04
438	Yellow	L	D	10	W	4.8	4	4	9.17
439	Yellow	W	D	10	W	5.3	4	4	8.89
440	Yellow	SN	S	10	W	5.8	0	0	9.04
441	Yellow	L	D	10	W	8	4	4	9.17
442	Yellow	SN	D	5	W	6.8	4	4	7.57
443	Yellow	L	S	10	W	6.5	4	4	7.83
444	Yellow	SN	S	5	E	7.3	4	4	7.56
445	Yellow	W	D	5	SW	9.4	4	4	7.57
446	Yellow	L	D	10	W	6	4	4	7.83
447	Yellow	W	D	10	W	4.1	4	4	9.20
448	Yellow	L	S	10	W	7	4	4	8.28
449	Yellow	W	U	10	W	4.1	4	3	9.06
450	Yellow	W	U	10	W	8.4	4	4	8.41
451	White	W	D	5	W	4.6	0	0	8.90
452	White	SN	S	10	W	9.2	4	4	9.03

453	White	SN	D	10	W	3.5	3	3	8.61
454	White	L	S	10	W	8.5	4	4	9.04
455	White	W	D	10	W	4.8	4	4	7.33
456	White	SN	D	10	W	6.2	4	4	9.22
457	White	W	D	10	W	7.8	4	4	7.91
458	White	W	D	10	W	5	4	-	8.90
459	White	W	D	10	W	5.6	4	4	9.37
460	White	L	S	10	W	3.4	4	00	9.22
461	White	W	D	10	W	3	4	2	8.47
462	White	SN	D	10	W	6.5	4	2	8.61
463	White	SN	U	10	W	8	4	4	9.24
464	White	L	S	10	W	7.5	1	00	9.24
465	White	W	S	10	W	6.7	4	4	9.32
466	White	SN	S	10	W	4	4	4	7.39
467	White	SN	S	5	W	7	4	4	8.89
468	White	SN	S	10	W	7.5	4	4	9.11
469	White	SN	S	10	W	5.8	4	4	8.34
470	White	SP	U	30	S	5	4	00	9.39
471	White	L	S	10	W	5.2	4	4	8.88
472	White	SN	D	10	W	4.2	4	4	8.61
473	White	SN	D	10	W	6.2	4	4	9.16
474	White	L	D	10	S	4.8	4	3	9.39
475	White	W	D	10	W	6.5	4	4	8.45
476	White	W	D	5	W	2.7	0	00	9.27
477	White	W	D	10	W	4.6	4	4	9.28
478	White	W	D	5	W	5.6	3	00	9.07
479	White	L	S	10	W	4.1	4	4	9.00
480	White	W	S	10	W	3.1	4	4	9.00
481	White	L	S	10	W	5.2	4	4	8.61
482	White	SN	D	10	W	7.5	4	4	9.00
483	White	SN	U	10	W	6.8	4	4	8.99
484	White	W	D	10	W	7.4	4	4	9.11
485	White	SN	D	10	W	3.7	4	4	9.04
486	White	SP	D	5	W	5.3	4	4	9.18
487	White	SN	D	10	W	4	4	4	9.00
488	White	W	D	10	W	3.3	4	4	9.01
489	White	L	D	5	W	4.5	4	4	9.18
490	White	W	S	10	W	7	4	4	8.91

491	White	L	S	5	W	7	4	2	9.35
492	White	SP	D	10	W	7	4	4	9.20
493	White	SN	S	10	W	5.9	3	00	9.04
494	White	SN	D	10	NW	9	4	4	7.69
495	White	L	D	10	S	4.3	4	3	9.39
496	White	W	D	10	W	5.5	4	-	9.04
497	White	L	U	10	NW	5.7	4	4	7.69
498	White	SN	S	20	S	5.3	4	4	8.22
499	White	W	S	10	W	8.1	4	4	8.45
500	White	W	S	5	W	6.5	4	4	9.18
501	Yellow	W	D	10	SW	2.1	4	4	9.02
502	Yellow	SN	D	20	W	9.8	4	4	9.14
503	Yellow	L	S	30	W	5.3	4	4	9.21
504	Yellow	SP	S	20	W	2.8	4	4	9.33
505	Yellow	SN	S	10	W	3.5	4	4	9.23
506	Yellow	W	U	20	W	4.4	4	4	9.23
507	Yellow	SN	S	10	W	6.1	4	4	9.20
508	Yellow	W	S	10	W	2.4	4	4	9.25
509	Yellow	W	S	10	W	3.6	4	2	9.26
510	Yellow	-	-	-	-	-	-	-	-
511	Yellow	-	-	-	-	-	-	-	-
512	Yellow	-	-	-	-	-	-	-	-
513	Yellow	-	-	-	-	-	-	-	-
514	Yellow	-	-	-	-	-	-	-	-
515	Yellow	W	S	20	NW	3.5	4	4	7.69
516	Yellow	W	S	10	W	4.8	4	4	7.69
517	Yellow	W	S	20	NW	6	4	4	7.98
518	Yellow	L	S	10	W	3.6	4	4	9.01
519	Yellow	W	D	10	W	9.1	4	4	9.20
520	Yellow	W	D	10	W	6.3	4	4	8.82
521	Yellow	SP	S	10	SW	5.2	4	3	9.11
522	Yellow	L	S	10	W	7.3	4	4	9.25
523	Yellow	SN	D	10	NW	6	4	4	8.18
524	Yellow	L	D	10	SW	4.3	4	4	8.48
525	Yellow	W	D	10	W	4.3	4	00	9.28
526	Yellow	W	D	10	W	5.5	4	4	9.26
527	Yellow	W	S	20	W	6.2	4	4	9.04
528	Yellow	SP	S	20	W	7.5	4	4	9.25

529	Yellow	W	S	10	W	5.2	4	00	9.00
530	Yellow	W	S	10	W	7.1	4	4	8.99
531	Yellow	W	D	10	W	6	4	4	9.01
532	Yellow	W	S	10	W	4.2	0	0	8.99
533	Yellow	L	S	10	W	5.8	4	4	8.28
534	Yellow	SN	D	30	W	5.1	4	4	9.06
535	Yellow	SN	D	10	W	7.1	4	4	9.89
536	Yellow	L	D	10	W	3	0	00	7.98
537	Yellow	SN	D	10	W	6	4	4	9.00
538	Yellow	W	S	10	W	5	4	4	8.96
539	Yellow	L	D	10	W	6	4	3	9.08
540	Yellow	SN	D	10	W	4.4	4	4	9.08
541	Yellow	W	D	10	W	4.7	4	4	8.91
542	Yellow	L	D	10	W	5.2	4	4	8.96
543	Yellow	L	D	10	W	2.6	4	3	8.85
544	Yellow	W	D	10	W	3.8	4	4	8.99
545	Yellow	SN	D	10	W	6	4	4	8.93
546	Yellow	W	D	10	W	7.5	4	4	9.49
547	Yellow	W	S	10	W	5	4	4	9.62
548	Yellow	L	D	10	W	6.3	4	4	8.96
549	Yellow	W	D	10	W	8.8	4	4	8.96
550	Yellow	L	D	10	W	6.1	4	4	8.96
551	White	-	-	-	-	-	0	00	-
552	White	L	S	10	W	5.2	4	00	9.00
553	White	-	-	-	-	-	-	-	-
554	White	W	D	10	W	4	3	3	8.95
555	White	SN	D	5	W	3	0	00	9.07
556	White	W	S	10	W	4.2	4	3	8.95
557	White	W	D	10	W	1.8	4	00	8.95
558	White	SN	D	10	W	6.2	4	3	9.00
559	White	SN	-	10	W	8.1	-	-	-
560	White	L	S	10	W	3.8	3	4	8.95
561	White	SN	S	30	NW	7.5	4	3	9.27
562	White	L	D	10	W	5.2	3	3	8.91
563	White	W	D	20	SW	8	3	4	7.39
564	White	SN	S	5	NW	6.5	4	4	9.60
565	White	W	D	10	SW	4.1	4	3	7.39
566	White	SN	S	10	SW	6.3	4	4	9.63

567	White	-	-	-	-	-	-	-	-
568	White	W	D	10	W	4	2	00	8.96
569	White	SN	U	10	W	6.6	4	4	8.95
570	White	W	S	10	W	7.2	4	4	9.14
571	White	L	D	10	W	5.2	4	4	9.14
572	White	L	D	10	W	6.1	4	4	9.47
573	White	SN	S	20	W	3.5	4	4	9.13
574	White	W	S	20	NW	3	1	00	9.25
575	White	W	S	10	W	4.4	4	4	8.91
576	White	W	S	10	W	5.2	4	4	8.91
577	White	W	S	10	W	4	4	2	8.89
578	White	SN	S	10	W	5.7	4	4	9.20
579	White	SN	S	10	W	5.6	4	0	9.20
580	White	SN	-	-	-	5.1	0	00	-
581	White	W	D	10	W	5.3	4	3	9.25
582	White	W	S	10	W	7.5	4	4	8.91
583	White	SN	S	10	NW	2.7	4	4	9.25
584	White	SP	S	10	W	4.2	4	4	9.02
585	White	W	S	20	W	1.5	3	2	8.73
586	White	L	S	10	W	7.1	4	2	8.87
587	White	W	D	10	W	9.5	4	4	7.83
588	White	W	S	10	W	3.1	3	3	9.23
589	White	L	S	10	W	6.2	4	4	9.27
590	White	SN	D	10	W	5.3	4	00	8.16
591	White	W	S	20	W	8	4	4	9.01
592	White	W	D	10	W	5.3	4	3	8.99
593	White	W	D	10	SW	5	4	4	9.20
594	White	L	S	20	W	7.1	3	4	9.14
595	White	L	S	20	NW	6.8	4	4	8.48
596	White	W	D	10	W	7.1	4	4	-
597	White	W	S	10	W	5.8	4	3	8.18
598	White	-	S	-	-	-	4	3	8.18
599	White	SN	S	10	NW	4.2	4	-	8.48
600	White	L	S	10	W	2.9	3	00	9.01
601	Yellow	W	D	5	W	4.1	4	3	9.57
602	Yellow	L	D	10	W	3.7	4	4	9.35
603	Yellow	SN	D	10	W	7	4	4	9.20
604	Yellow	W	S	10	W	9.9	4	4	9.20

605	Yellow	L	S	10	W	7	4	4	9.22
606	Yellow	L	D	10	W	3.8	4	4	9.20
607	Yellow	SN	S	10	W	5.1	4	4	9.52
608	Yellow	L	U	10	W	7	4	4	8.85
609	Yellow	SN	S	10	W	7	4	4	9.20
610	Yellow	W	S	10	NW	3.4	4	2	9.33
611	Yellow	SN	S	10	W	4.4	4	4	9.21
612	Yellow	W	S	10	W	7.9	4	00	9.34
613	Yellow	-	-	-	-	-	-	-	-
614	Yellow	W	D	5	W	7.5	4	4	9.56
615	Yellow	L	D	20	NW	4	4	3	9.00
616	Yellow	SN	D	10	W	5.2	4	4	8.93
617	Yellow	L	D	10	NW	8.3	4	3	8.85
618	Yellow	L	D	10	W	6.2	0	00	8.85
619	Yellow	W	D	10	W	7.8	4	4	8.95
620	Yellow	W	D	10	W	9	4	4	8.16
621	Yellow	W	U	10	SW	4.2	4	4	9.39
622	Yellow	SN	S	10	W	5	4	4	8.34
623	Yellow	W	D	10	S	5.4	4	4	9.20
624	Yellow	W	D	10	W	6	3	3	9.05
625	Yellow	SN	S	30	NW	7.6	4	4	8.94
626	Yellow	W	S	10	W	5.8	3	3	9.05
627	Yellow	SN	D	10	W	5.8	4	4	9.16
628	Yellow	W	S	10	W	4.7	3	4	8.99
629	Yellow	SN	S	10	W	7.2	4	4	9.16
630	Yellow	L	U	10	W	8.5	4	4	8.99
631	Yellow	W	D	10	W	7	4	4	9.01
632	Yellow	W	D	10	W	8.3	4	4	8.93
633	Yellow	SN	S	5	W	6	4	4	9.34
634	Yellow	L	D	10	NW	8.3	4	4	9.33
635	Yellow	SN	S	10	W	5.5	4	4	9.47
636	Yellow	L	D	10	W	8.6	4	4	9.52
637	Yellow	-	-	-	-	-	0	00	-
638	Yellow	W	U	10	W	4.7	4	4	9.65
639	Yellow	SN	S	10	W	6.4	3	3	9.56
640	Yellow	SN	D	10	W	6	4	00	9.66
641	Yellow	W	D	10	W	3.1	4	4	9.52
642	Yellow	W	D	10	W	8.3	4	4	8.56

643	Yellow	W	D	10	W	8	4	4	8.92
644	Yellow	-	-	-	-	-	-	-	-
645	Yellow	L	S	10	W	4.8	4	4	7.44
646	Yellow	W	S	10	W	7.4	4	4	7.39
647	Yellow	W	D	10	W	6.5	4	4	7.33
648	Yellow	W	D	10	W	7.2	4	4	7.34
649	Yellow	L	D	5	W	3.2	4	3	9.18
650	Yellow	L	S	10	W	6	4	4	7.84
651	White	SN	D	10	W	6.5	4	4	9.20
652	White	W	S	10	W	5	4	4	8.87
653	White	W	S	10	NW	8.3	4	4	8.48
654	White	SN	U	30	NW	7.1	4	4	8.94
655	White	-	-	-	-	-	-	-	-
656	White	L	S	20	W	8.2	4	4	8.48
657	White	-	-	-	-	-	-	-	-
658	White	-	-	-	-	-	-	-	-
659	White	L	S	10	W	6.3	4	4	8.87
660	White	-	-	-	-	-	-	-	-
661	White	L	S	10	SW	4.2	4	2	8.87
662	White	-	-	-	-	-	-	-	-
663	White	L	S	10	W	5.5	4	3	9.20
664	White	SN	U	10	W	5	4	4	7.56
665	White	L	S	10	W	7.6	4	4	8.28
666	White	W	S	5	SW	4.2	4	4	7.57
667	White	L	D	10	W	6	4	4	8.39
668	White	W	S	10	W	6.2	4	4	7.56
669	White	SN	S	10	NW	5.4	4	4	8.41
670	White	SN	-	10	W	8.2	-	-	-
671	White	SN	D	10	W	9.9	4	4	7.83
672	White	W	S	5	W	7.5	4	4	9.35
673	White	SP	S	20	W	4.1	4	4	9.06
674	White	W	S	10	W	5.4	4	4	9.04
675	White	SN	D	10	W	6.8	4	4	8.95
676	White	SN	D	5	W	4.5	4	4	9.54
677	White	SN	S	5	W	6.2	4	4	9.56
678	White	W	D	20	W	3	1	00	8.97
679	White	SN	U	20	W	7	4	4	8.47
680	White	SN	D	10	W	6	4	4	9.16

681	White	L	S	10	W	3.5	4	4	9.01
682	White	SN	U	5	W	5.6	4	4	9.56
683	White	SN	U	10	W	3	4	4	9.21
684	White	SN	S	10	NW	3	4	4	9.33
685	White	SN	U	5	W	4.6	4	4	9.56
686	White	W	D	10	W	4.7	4	4	9.52
687	White	L	S	10	W	1.5	0	00	8.85
688	White	L	S	10	W	1	4	2	8.90
689	White	SN	S	10	W	3.8	4	4	8.90
690	White	L	S	10	W	3.3	4	0	8.90
691	White	W	S	10	NW	9	4	3	9.34
692	White	SN	S	10	SW	3.7	4	4	9.34
693	White	W	D	10	W	5.4	4	4	9.33
694	White	L	S	10	W	3	4	3	9.49
695	White	SN	D	10	W	5.8	4	4	9.33
696	White	L	S	10	W	3.5	0	00	9.49
697	White	W	U	10	W	5.7	4	4	9.09
698	White	W	D	20	W	6	4	4	8.97
699	White	L	S	10	W	3	4	3	9.49
700	White	L	D	10	W	6	4	2	8.85
701	Yellow	W	D	10	W	8.9	4	4	9.89
702	Yellow	W	S	10	NW	4.3	4	2	9.34
703	Yellow	W	S	10	W	5	4	4	9.03
704	Yellow	L	D	10	W	4	4	4	9.39
705	Yellow	SN	S	10	W	3.2	4	4	8.74
706	Yellow	W	S	10	SW	4.8	4	00	8.74
707	Yellow	SN	S	10	W	5.7	4	2	8.74
708	Yellow	W	D	10	W	6.3	4	4	9.39
709	Yellow	L	S	10	W	4.6	4	0	9.00
710	Yellow	L	S	20	NW	5.2	4	4	8.94
711	Yellow	W	-	-	-	3	-	-	-
712	Yellow	W	S	30	NW	7	4	4	8.94
713	Yellow	L	S	20	SW	6	4	00	9.47
714	Yellow	SN	D	10	W	6	3	00	9.04
715	Yellow	W	S	10	W	4	4	4	9.47
716	Yellow	L	-	5	W	6.3	-	-	-
717	Yellow	SN	D	10	W	6.4	4	4	9.49
718	Yellow	L	-	5	W	2.8	-	-	-

719	Yellow	W	D	10	W	6	4	4	9.57
720	Yellow	SN	U	10	W	5.2	4	4	9.57
721	Yellow	L	S	10	W	8.8	4	4	8.87
722	Yellow	SN	S	10	W	7.3	4	4	9.14
723	Yellow	SN	S	10	W	7	4	4	8.87
724	Yellow	SN	U	10	W	7.3	4	4	9.14
725	Yellow	SN	S	10	W	11.1	4	4	8.90
726	Yellow	SN	D	10	W	4.6	4	4	7.98
727	Yellow	SN	S	10	W	6	4	4	7.91
728	Yellow	SN	S	10	W	7.3	4	4	9.28
729	Yellow	W	D	10	W	9	4	4	9.11
730	Yellow	W	S	10	W	6.3	4	4	9.11
731	Yellow	SN	S	10	W	4.9	4	4	9.14
732	Yellow	L	S	10	W	8.2	4	4	9.24
733	Yellow	L	S	10	W	8.9	4	4	9.24
734	Yellow	W	S	10	NW	6.2	4	3	9.01
735	Yellow	L	D	10	W	6.9	3	4	8.98
736	Yellow	W	S	10	W	4	4	4	8.99
737	Yellow	W	U	10	W	5	4	4	9.20
738	Yellow	W	S	10	NW	3.6	4	4	8.99
739	Yellow	SN	D	10	NW	4	4	4	8.99
740	Yellow	SN	D	10	W	3.5	4	4	9.20
741	Yellow	L	D	10	W	11.5	4	3	8.98
742	Yellow	W	S	10	W	4.4	4	4	9.07
743	Yellow	W	S	10	W	5	4	4	8.95
744	Yellow	W	S	10	NW	7.9	4	4	9.16
745	Yellow	W	S	20	NW	11.5	4	4	8.47
746	Yellow	W	D	20	SW	4.3	4	3	9.01
747	Yellow	L	S	10	W	9	4	4	9.32
748	Yellow	L	S	10	W	6.3	4	4	8.91
749	Yellow	SN	U	10	W	6	4	4	8.87
750	Yellow	SN	D	10	W	6.4	4	4	8.74
751	White	L	S	10	W	1.3	0	00	8.87
752	White	W	S	10	W	6.1	4	4	9.47
753	White	L	S	10	W	5	4	3	9.47
754	White	E	S	10	W	4.3	3	1	9.47
755	White	SN	D	10	W	7.9	4	3	8.74
756	White	L	S	10	W	5	4	4	9.03

757	White	W	D	5	W	3.5	1	00	9.44
758	White	SP	S	10	W	2.9	2	00	9.20
759	White	SN	U	10	W	1.8	4	3	9.19
760	White	L	S	10	W	4.1	4	4	8.34
761	White	W	D	20	NW	4.9	4	4	9.16
762	White	L	S	20	W	4.5	4	4	9.20
763	White	L	S	10	W	4	4	4	8.71
764	White	W	S	10	NW	3	4	2	8.73
765	White	W	S	5	W	7.5	4	4	9.60
766	White	W	D	10	W	6.4	4	00	9.89
767	White	W	-	10	W	3	-	1	-
768	White	W	D	10	W	5	4	00	9.56
769	White	SN	D	20	SW	4.5	4	4	9.63
770	White	SN	D	10	W	5.7	3	00	9.14
771	White	SN	S	10	W	3.8	4	4	9.89
772	White	W	S	10	W	5.6	4	4	9.00
773	White	W	S	10	W	3.7	4	4	9.47
774	White	SN	S	20	W	3.1	4	3	9.06
775	White	W	S	10	W	3.8	4	4	9.13
776	White	W	S	20	S	3.3	4	3	9.06
777	White	L	D	10	W	5.1	4	4	9.27
778	White	L	S	10	W	5.7	4	4	9.22
779	White	W	S	30	NW	4	4	3	9.06
780	White	L	S	10	W	7.6	4	4	7.44
781	White	L	D	10	W	10	4	4	8.18
782	White	SN	D	10	W	6	4	4	8.56
783	White	W	S	10	W	3.6	4	4	7.34
784	White	W	S	10	W	4.9	4	4	7.84
785	White	L	D	10	W	11.2	4	4	8.33
786	White	SN	U	10	W	5	4	3	9.07
787	White	SN	-	20	NW	7	-	4	-
788	White	W	D	10	SW	3	4	00	9.09
789	White	W	D	10	W	5.2	4	3	9.13
790	White	SN	D	10	W	7	4	4	-
791	White	L	S	10	W	5.9	4	3	9.32
792	White	W	D	10	W	3.9	4	00	9.56
793	White	SN	D	10	W	4.9	4	4	9.52
794	White	SN	S	10	W	5.3	4	4	9.14

795	White	SN	D	5	W	5.6	4	4	9.54
796	White	W	S	10	W	4	4	2	9.51
797	White	SN	U	10	W	3.7	4	4	9.39
798	White	W	S	10	W	4	4	4	9.14
799	White	W	D	5	W	4.7	4	3	9.47
800	White	W	S	10	W	4.2	4	4	9.14
801	Yellow	W	D	20	W	7.1	4	4	9.11
802	White	E	NA	20	SW	10	4	4	8.28