IMPACT OF SEVERE FIRE ON ECTOMYCORRHIZAL FUNGI OF WHITEBARK PINE SEEDLINGS

by

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TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

Table Page

LIST OF TABLES CONTINUED

Table Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES CONTINUED

Figure Page

ABSTRACT

Whitebark pine (*Pinus albicaulis*) is a threatened keystone species in subalpine zones of Western North America critical to watersheds and maintenance of high elevation biodiversity. Pine nuts are an important food for wildlife including grizzly bears. Whitebark pine stands have experienced losses up to 90% due to white pine blister rust, mountain pine beetles and replacement due to fire suppression. Active management strategies include letting natural fires burn or applying prescribed fires to clear understory fir, stimulate seedling regeneration and provide openings for nutcrackers to plant seeds. However, post-fire plantings of rust-resistant seedlings have low survival rates. This study evaluated the impact of fire on the mycorrhizal fungi which are obligate mutualists with whitebark pine and to address management concerns. The 2001 Fridley fire burned a portion of a mature whitebark pine forest and a year later 20,000 seedlings were planted. After four years, natural and planted seedlings, on the burn and controls in the adjacent unburned forest were well colonized by mycorrhizal fungi (>90%) although a portion may be nursery E-strain. The severe burn reduced mycorrhizal diversity 27% on natural and planted seedlings and caused a significant shift in mycorrhizal species (determined by ITS sequencing, principal component analysis and multidimensional scaling). Seedlings in the burn (natural and planted) were dominated by *Pseudotomentella nigra, Wilcoxina* species and *Amphinema byssoides* while natural seedlings in unburned forest hosted mainly *Cenococcum geophilum* and *Piloderma byssinum*. Differences were minimal between planted and natural seedlings in the burn, but roots of planted pines retained the container shape. The functional significance of a species shift to seedling survival is not yet known. Seedlings in all treatments hosted suilloid fungi (*Rhizopogon, Suillus*) important in pine establishment. A greenhouse bioassay of burned and unburned soils using nursery seedlings did not reflect the full diversity found in the field study, but did reveal suilloid fungi indicating that bioassays can be used as a pre-planting assessment tool for this group. Despite high mycorrhization and availability of suilloids, seedling survival was low (22-42%) suggesting the timing/type of mycorrhization and/or other biotic/abiotic factors are a concern.

CHAPTER 1

LITERATURE REVIEW

Synopsis

Whitebark pine is a highly threatened keystone tree species in the subalpine zone of western and northwestern North America. Because of its importance and the large reduction in its population due to various factors, intensive restoration efforts have been initiated. A treatment considered key to restoration of whitebark pine forests is fire and the planting of seedlings. However, fire can potentially alter the microbial community in the soil, in particular the community of obligate mutualists (ectomycorrhizal fungi) on whitebark pine roots, resulting in unsustainable systems rather than the desired restoration of whitebark pine forests. It is important to examine the impact of fire on the ectomycorrhizal fungi associated with whitebark pine to fully understand how this complex system is maintained, and in order to develop the most comprehensive restoration plan possible.

Whitebark Pine

Pinus albicaulis Engelm. (whitebark pine) is the only North American species of stone pine: family *Pinaceae*, subgenus *Strobus*, and subsection *Cembrae* (Price et al. 1998). Whitebark pine and stone pines in general are characterized by five needle fascicles, indehiscent cones, and large wingless seeds dispersed by an avian agent, which is the Clark's nutcracker for whitebark pine (Price et al. 1998, McCaughey and Schmidt

2001). The western range of whitebark pine is within the subalpine zone in the Rocky Mountains of southwestern Canada, the Cascade and Coastal Ranges of Washington and Oregon and south to the Sierra Nevada Mountains of California, and in small areas of Nevada. To the east whitebark pine exists near tree-line in the northern Rockies of Idaho, Montana, and Wyoming (McCaughey and Schmidt 2001). In areas of higher moisture, whitebark pine coexists as a seral species with other subalpine tree species such as Engelmann spruce and subalpine fir (McCaughey and Schmidt 2001, Weaver 2001). In drier environments, whitebark pine is a climax species forming pure or nearly pure stands (Arno 2001, Weaver 2001). At tree-line, whitebark pine often assumes a twisted shrub-like form called krummholz.

Whitebark Pine as a Keystone Species:

In its subalpine habitat, whitebark pine provides a number of valuable ecosystem functions that help define it as a keystone species. Two are primarily responsible for this designation: its role in increasing the diversity of organisms that can survive in these subalpine areas and its importance in maintaining healthy watersheds (Tomback et al. 2001, Primack 2002, Schwandt 2006).

Whitebark pine increases the number of plant and animal species able to inhabit the subalpine through two main mechanisms. The first is by initiating succession. The environment in the upper subalpine and after fires is incredibly harsh. Typically, these areas have more sun exposure, less water retention, and lower nutrient availability than many plants can withstand (Hansen-Bristow et al. 1990). The temperature of typical whitebark pine habitat in January fluctuates between -5° and -14°C and in the summer

months, rainfall can be as minimal as 4 mm (Weaver 2001). Whitebark pine counters these obstacles to its establishment with a bird dispersal agent and hearty seeds that allow it to be an early colonizer of harsh habitats and to survive where other plants could not (Tomback et al. 1993). After whitebark pine is established, more hospitable microhabitats develop around the trees allowing other plants and animals to institute themselves (Lanner and Vanderwall 1980).

A second way whitebark pine increases diversity in the subalpine is through production of large meaty seeds that are an important nutritional component of many animals' diets. The seeds are an important food source for red squirrels, Clark's nutcracker (whitebark pine's dispersal agent), and the endangered grizzly bear (Mattson et al. 1992, Mattson 2000, Mattson et al. 2001). The seeds of whitebark pine are an essential component of the pre-hibernation diet of grizzly bears and biologists are certain bears will suffer additional losses in their already low numbers if seeds are not available (Kendall and Arno 1990, Mattson et al. 2001).

In addition to its importance in fostering biodiversity, whitebark pine is critical in watershed dynamics (Farnes 1990). *Pinus albicaulis* is often the highest elevation tree species where it exists and typically delineates tree line. The large spreading canopies of whitebark pine contribute to a persistent snow-pack and retarded snow melt in spring resulting in more consistent run-off in summer. The physical presence of the trees and their root systems along with slower melt-off rate decreases erosion of the poorly developed topsoil in subalpine zones, also resulting in lower particulate matter in the water downstream (Farnes 1990).

Threats to Whitebark Pine:

The invaluable ecosystem services that whitebark pines provide are in danger of being lost as the species experiences severe reductions in numbers: 40-90% over its entire range (Tomback et al. 2001). Three main agents have brought about massive declines in whitebark pine communities: fire suppression, white pine blister rust, and mountain pine beetles.

Mountain pine beetle (*Dendroctonus ponderosae* Hopkins) is a native parasite of whitebark pine and can pose a serious threat to the tree. Mountain pine beetle attacks typically cause mortality in older solitary trees and small tree clusters. However, mountain pine beetle populations can also reach epidemic levels causing large-scale decimation of whitebark pine (Ciesla and Furniss 1975, Kegley et al. 2001).

For tree populations as a whole, white pine blister rust (*Cronartium ribicola*) has been considered a more serious danger to *P. albicaulis* trees than the mountain pine beetle. White pine blister rust was introduced to North America from Europe, and whitebark pine trees exhibit little natural resistance (1-5% of trees) to this foreign invader (Hoff and Hagle 1990, Hoff et al. 2001). Where white pine blister rust occurs it is coupled with high infection rates often reaching near 100% (Hoff et al. 2001). White pine blister rust kills small trees outright and kills the upper canopy branches of larger trees before they too succumb. The death of the upper branches restricts cone production and eliminates its ability to reproduce.

White pine blister rust has a complex life cycle and it requires specific conditions to complete reproduction and to spread (Hoff et al. 2001). In addition to the white pine

hosts, this rust needs a *Ribes* species in reasonable proximity to serve as the alternate host, without which the rust cannot spread (Hahn 1928). The infectious spores that travel between the *Ribes* and white pine hosts are sensitive, requiring specific environmental conditions to maintain viability (Mielke 1943). These environmental constraints result in a high concentration of rust infection in the Pacific Northwest portion of its range, and in the northern U.S. and Canadian ranges west of the continental divide where weather is more conducive to infection (Gynn and Chapman 1951, Keane and Morgan 1994, Kendall 1999). However, these environmental restrictions have not stopped infection into regions with unfavorable climatic conditions for rust infection (Hoff and McDonald 1993, Smith 1990, Smith 1997).

Fire exclusion is considered another major factor in the loss of whitebark pine communities. Historically, natural fires have been a key component in maintaining whitebark pine forests. When fire burns a forest, whitebark pine is more likely to survive than its competitors Engelmann spruce (*Picea engelmannii*) and subalpine fir (*Abies lasiocarpa*). In addition, whitebark pine is a stronger competitor in the newly opened understory. The openings created by fire also provide new areas for caching of whitebark pine seeds by Clark's nutcrackers. Through fire and caching, whitebark pine is able to avoid successional replacement by its competitors (Arno 1986).

Fire suppression has been the *de facto* management technique for most public and private lands since the early 1900s (Keane and Arno 2001). For whitebark pine communities that are adapted to a moderate to severe burn every 50 – 400 years, fire exclusion appears to have had disastrous effects (Arno 1980, Arno 1986, Romme 1982,

Barrett 1994). Fire suppression allows the shade tolerant competitors of whitebark pine, such as Engelmann spruce and subalpine fir to increase and eventually dominate in seral whitebark pine stands. Given time, these competitors can exclude recruitment of whitebark pine seedlings, which are more shade intolerant. Given enough time, shade-tolerant species will locally replace whitebark pine (Minore 1979, Arno and Hoff 1990, Keane and Morgan 1994).

Individually, rust, beetles, and fire suppression have a detrimental effect on whitebark pine, but they are not isolated from each other in nature, and when they act upon trees synchronously the damage is compounded. Areas where the pine is seral and thus susceptible to losses due to advanced succession are also the areas most prone to white pine blister rust (Kendall and Keane 2001). When whitebark pine is stressed from competition with other subalpine trees in advanced succession or by rust infection, trees are more susceptible to attack and mortality from mountain pine beetles (Arno 1986). Also, without fire, the density of mature trees is higher, making the forest more prone to beetle attacks, since beetles preferentially infect large older trees (Kendall and Keane 2001). In addition, a lack of fire in rust areas prevents new openings for seedlings to establish, and ultimately prevents the possibility of a rust resistant strain from developing naturally.

The recent losses of whitebark pine trees have been astronomical, and the hardest hit areas are those most prone to rust infection and advancing succession. For example, in the Interior Columbia River Basin and Big Hole region of Southwest Montana, there has been an almost 50% reduction in the area occupied by whitebark pine in the last 90 years

(Keane et al. 1996a). Smaller areas with the environment most conducive for rust spread and infection have experienced losses of whitebark pine up to 98% (Keane et al. 1996b). These losses can happen incredibly quickly and western Montana has lost 42% of its whitebark pine in the last 20 years (Keane and Arno 1993). These rapid losses paint a bleak picture for whitebark pine and the situation is made even more dire as whitebark pine does not produce female cones until it is at least 65 years old (McCaughey and Schmidt 1990, McCaughey and Schmidt 2001).

Restoration of Whitebark Pine:

Due to the acknowledged importance of whitebark pine to subalpine ecosystems, active attempts are being made to restore these forests. Many management techniques are currently being employed in an attempt to bolster populations of whitebark pine (Keane and Arno 1996, Keane et al. 1996a).

Ribes Eradication:

The first attempts to protect these trees were made in the early nineteen hundreds when the main threat to whitebark pine and other white pines was white pine blister rust (Tomback et al. 2001). This initial program attempted to limit the spread of rust by elimination of the required alternate host, *Ribes*. This effort began in 1921 and thousands of effort-hours were spent mechanically removing and spraying *Ribes*, the alternate host of white pine blister rust. This attempt was cancelled in 1966 and considered a failure due to the tenacious nature of *Ribes*, which has the ability to resprout from roots, and long lasting seeds (Hoff et al. 2001).

Rust-Resistance Breeding:

A rust-resistance breeding program was initiated for white pines that ran concurrent to the *Ribes* eradication attempt. Trees that displayed no signs of infection were located in areas that had been decimated by the rust (Lachmund 1934, Mielke 1943). Seeds were harvested from these promising survivors in the hopes that their rust-resistance would be highly heritable (Bingham 1983). The general conclusion is that the resistance conferred to the progeny is variable, and results were mixed (Hoff and McDonald 1980). This conclusion made for white pines has been extended to whitebark pine as this species is assumed to have similar population genetics. However, today there are several nurseries growing these costly whitebark pine seedlings, including the USDA Forest Service nursery in Coeur D'Alene Idaho and Dorena Genetic Resource Center in Cottage Grove, OR. Since 1990, 200,000 rust resistant whitebark pine seedlings have been planted in the Rocky Mountains, many in national forests and some in parks such as Yellowstone and Glacier (Hoff et al. 2001). The loss of genetic diversity in these seedlings is also being evaluated (Mahalovich and Dickerson 2004).

Current Efforts Using an Integrated Approach:

Today, as the situation regarding whitebark pine has become increasingly dire, the restoration effort has intensified and now takes an integrated approach. A main component in the integrated approach for restoring and managing whitebark pine forests is reestablishment of a historical fire regime (Keane and Arno 2001). This includes the reintroduction of fire with prescribed burning and implementation of a policy that allows natural burns to run their course. The return to a more natural fire regime is thought to

promote the health and restoration of whitebark pine through many different avenues. Fire opens both the understory and the canopy and provides openings for seed caching by birds and light for the shade-intolerant whitebark pine seedlings so that they can once again become established (Hutchins and Lanner 1982, Tomback 1982). In addition, it is thought that fire may help prevent the spread of beetles because resulting forests have mixed-age diversity and beetles preferentially attack older trees (Keane et al. 1990, Hoff et al. 2001). Rust infection has been found to increase as time since the last fire increased (Moody 2006).

Many other restoration techniques are being used, singularly or in combination with fire for integrated management. One practice is to selectively log competitor tree species and/or clear-cut small areas. Logging provides open space for seed caching and enough light for seedlings to establish, plus it slows succession. Logging is also used to control fuel loads so that the severity of a prescribed burn can best match the managers' plan. Another practice is the planting of rust-resistant whitebark pine seedlings after burning (Keane and Arno 2001). This practice gives whitebark pine seedlings an establishment advantage over competitors and a possible defense against blister rust.

The Bitterroot RWPE Project:

The implementation and testing of integrated techniques to restore whitebark pine forests has been initiated. The Restoring Whitebark Pine Ecosystems (RWPE) project headed by Robert Keane and the Rocky Mountain Research Station is currently spearheading the restoration effort (Keane and Arno 1996, Keane et al. 1996a, Keane and Arno 2001). This large-scale project involves numerous whitebark pine forests that differ

in multiple factors such as rust infection levels, stand makeup, and other abiotic and biotic factors (Keane and Arno 1996; Keane et al. 1996a). Various combinations of restoration treatments are being applied to the whitebark pine stands to determine the most effective method for returning them to their original structure and ecological function. The goal of this project is to provide a useful set of guidelines for managers attempting to restore whitebark pine forests and to experimentally test these procedures. This includes determining which forests are in the most dire need of restoration, elucidating the role of whitebark pine in the forest/landscape, understanding pertinent fire regimes, and evaluating how succession status, mountain pine beetles, and white pine blister rust have affected the system and how they might affect the restoration effort (Keane and Arno 2001). To achieve this goal, it is necessary to understand how historical whitebark pine forests functioned and how these forests have been altered.

Thus far there are limited results from the RWPE project. Many of the selected whitebark pine forests slated for fire reintroduction as part of the management plan have not yet been burned due to the difficulties of using fire as a restoration tool. Both prescribed burning and letting natural wildfires run their course pose logistical problems. The main issue with any burn is keeping it under control. For prescribed burns, only specific environmental conditions assure that the fire will be contained to the designated area and it is often difficult to meet these conditions (Keane 2000, Keane and Arno 2001). Wildfires are even more difficult to control as they typically start at lower elevations and burn up to the subalpine. Wildfires that initiate at lower elevations are not being allowed to continue without suppression. This is due to human encroachment into

the wooded areas so low elevation wildfires are therefore extinguished to protect property (Keane and Arno 2001). In addition to control problems, 49% of whitebark pines' range is in wilderness and roadless areas, making it inaccessible to silvicultural techniques or prescribed burning. In 1998-1999 some of the plots in the RWPE project were burned and results in regard to restoration of whitebark pine are still being examined (Keane and Arno 2001).

Along with the RWPE project the regeneration of whitebark pine in Yellowstone National Park is being observed in areas burned in the 1988 wildfires as another opportunity to study the establishment of whitebark pine after fire. However, results are still minimal as whitebark pine is a very slow growing and maturing tree. These forests have also not been substantially exposed to blister rust and their ability to survive or resist infection is not known (Tomback et al. 1995, Mahalovich and Eramian 2000, Hoff et al. 2001, Mahalovich and Dickerson 2004).

Concerns:

Currently, multiple experiments are being carried out to determine which restoration treatment, or combination thereof, will be the most effective in restoring and/or maintaining whitebark pine forests. These studies are examining how factors such as advanced succession, blister rust infection rates, and mountain pine beetle infestation alter the effectiveness of the restoration treatments in whitebark pine communities (Tomback 1995, Keane and Arno 1996, Keane et al. 1996a). While it is logical to expect that factors such as advanced succession and high levels of rust infection or mortality will have an impact on the restoration of whitebark pine forests, what may be overlooked are

the unintended consequences of the restoration treatments themselves. One aspect that warrants examination is how restoration treatments, in particular fire, affect the soil microbial community, and mycorrhizae in particular. The natural ecology of whitebark pine forests has been altered by fire suppression and white pine blister rust, making it difficult to predict how the return of fire will impact systems. Fire suppression typically results in increased fuel loads, which can lead to higher severity fires. This can result in the wholesale elimination of whitebark pine trees along with their less fire-tolerant competitors, as was seen in the Smith Creek research site prescribed burn in western Montana, part of the RWPE (Keane and Arno 2001). Increased burn severity can also impact the soil microbes and the mycorrhizal community by increasing the depth and heating of the soil. This in turn can result in a reduction or loss of potential fungal partners for the reestablishment of seedlings after the fire. In addition, the loss of all reproductive whitebark pine trees, due to more severe burns or mortality due to rust, can result in the loss of seed source for regeneration. In this case, fire would need to be paired with planting, at the minimum, for successful reforestation.

Microbes and Mycorrhizal Fungi

Microscopic below-ground organisms have a direct effect on the aboveground flora. While not observable themselves, these cryptic organisms play a major role in influencing the plant community make-up, forest health and plant survival. Mycorrhizal fungi in particular have direct and indirect effects on soil structure, nutrient cycling, mutualistic and parasitic interactions, plant health and nutrition, and water relations in the

rhizosphere (Neary et al. 1999). This in turn influences the plant and animal community in a given area and makes an understanding of the composition and ecology of the mycorrhizal community an important aspect of restoration. When working with coniferous trees, the ectomycorrhizal fungi are an important group of rhizosphere microorganisms to consider.

Ectomycorrhizae

Pines are obligate mutualists with ectomycorrhizal fungi and normal growth does not occur without them (Smith and Read 1997, Read 1998). Ectomycorrhizal fungi form on the fine roots of their hosts, creating a sheath of hyphae around them called a mantle (Brundrett et al. 1996). Inside the roots, hyphae penetrate between the cortical cells creating a fungus/plant exchange interface (Allen 1991). The fungal hyphae branch out into the soil essentially increasing the surface area for absorption of various nutrients and water. The ectomycorrhizal fungi associated with any temperate tree species are an important consideration in restoration.

Ecology of Ectomycorrhizae:

Ectomycorrhizal fungi play multiple ecological roles that depend on the specific fungi, the host species, and the environmental conditions involved. A single host in nature typically supports multiple species of ectomycorrhizal fungi that may have different ecological specialties, and a forest type is supported by a particular mycorrhizal community. The most well understood function of ectomycorrhizal fungi is their ability to increase the availability and uptake of phosphorus and nitrogen to its host (Allen

1991). Some fungal species are able to increase the uptake from inorganic sources of these molecules, while others can also access organic sources. In addition, it has been recently discovered that some fungi have particular enzymes that allow them to use chemical weathering of rock and soil particles to attain P and N. Some species of ectomycorrhizal fungi are able to protect their host from pathogens (Perrin 1985a, Perrin 1985b, Morin et al. 1999). This can be accomplished physically, with the ectomycorrhizal sheath forming a protective barrier against root pathogens, or indirectly with the ectomycorrhizal fungi increasing the overall health of the host so it is able to resist pathogens (Allen 1992). The extensive mycelial network of ectomycorrhizal fungi can confer drought resistance to its host (Graham et al. 1987, Nardini et al. 2000, Landhausser et al. 2002). These benefits have been shown to be critically important to the host, especially in environments where the host species is stressed by limited nutrient or water availability such as treeline or alpine habitats (Gardes and Dahlberg 1996).

Host Specificity and Succession:

Ectomycorrhizal relationships differ in their levels of host-fungus specificity, which is influenced by multiple biotic and abiotic factors (Smith and Read 1997). Some ectomycorrhizal relationships are highly specific and a fungus may colonize only one or a few host species, as is true for suilloid fungi, which are restricted to members of the Pinacae, and mostly pines (Bruns et al. 2002, Mohatt 2006). Alternatively, some ectomycorrhizal fungi, such as *Cenococcum* are considered generalists that are able to infect and colonize a wide range of host species. Some host trees are only colonized by a few fungal species as is true for alder, while Douglas-fir can host hundreds of species.

The distribution of fungi is further complicated by a multitude of abiotic factors such as soil moisture, pH, temperature, and structure all of which influence the ability of a particular mycorrhizal fungus to survive (Cripps 2001). These abiotic factors can vary on a fine scale and when paired with biotic factors results in a distribution of ectomycorrhizal species that is typically patchy (Cripps and Eddington 2005). Another important concept in ectomycorrhizal relationships is that a successional dynamic is also functioning. The general paradigm is that young seedlings typically accept a range of generalist ectomycorrhizal fungi but as the trees mature the fungal relationship shifts towards fungi more specific to the host (Trappe 1977, Molina and Trappe 1982, Allen 1992). All of these ecological functions ultimately influence the health and survival of the tree.

Ectomycorrhizae and Fire:

Both fire and ectomycorrhizal fungi are critically important to the health and maintenance of whitebark pine forests. However, while there are numerous studies examining the aboveground influence of fire on whitebark pine populations and for restoration (Tomback et al. 1995, Keane and Arno 1996a, Keane and Arno 2001, Waring and Six 2005), there is no information on how fire impacts the ectomycorrhizal community belowground. Although whitebark pine and its fungal associates have evolved with fire, the historical ecology has been altered by fire suppression and possibly by climate change. A complete knowledge of how fire affects the ectomycorrhizal fungi associated with whitebark pine, especially in fire suppressed systems, will help managers choose the appropriate management technique to restore these forests.

The impact of fire on the ectomycorrhizal community of other tree species has been shown to be unpredictable, and results vary from no change to complete destruction of the fungi. One important variable may be burn severity (Neary et al. 1999, Cairney and Bastias 2007). Burn severity is influenced by fuel loads, temperature and moisture of the soil, and duration of the burn. However, comparisons are difficult as measuring burn severity is subjective and there are no set guidelines or definitions (Jain 2004). Results are typically relative to the system being examined. The relative nature of burn severity measurements makes it difficult to extrapolate the influence of burn severity on ectomycorrhizae from one system to another.

In addition, it is not easy to identify and quantify the mycorrhizal community. However, some studies report large shifts in species abundances, decreases in diversity, large losses in ectomycorrhizal biomass, and even complete loss of ectomycorrhizal species after severe fire (Horton et al. 1998, Baar et al. 1999, Grogan et al. 2000, Treseder et al. 2004). After less severe burns, results vary from no changes to decreases in ectomycorrhizal evenness, losses in ectomycorrhizal biomass, and decreases in species richness (Jonsson et al. 1999b, Stendell et al. 1999, Chen and Cairney 2002, Smith et al. 2004, Tuininga and Dighton 2004, de Roman and de Miguel 2005, Hart et al. 2005a, Hart et al. 2005b).

The problems in assessing the impact of fire on the mycorrhizal community are compounded by the difficulty of making comparisons across different types of forests. Forest systems are complex and the differences in diversity, species makeup, age,

structure, soil, climate, recent weather, geography, and innumerable other variables can confound comparative analysis.

Fire can influence the ectomycorrhizal community in a number of ways. If a burn is severe enough to kill the host plants this leads to a direct reduction in the ectomycorrhizal fungi that depend on these plant species. As burn severity increases, so does the depth of soil sterilization. Even a light burn can completely consume the litter and/or organic layer of the soil where a majority of ectomycorrhizae are located (Harvey et al. 1976, Harvey et al. 1979, Buchholz and Gallagher 1982). Fires can also affect soil pH, nutrient availability, moisture, temperature, and chemistry, all of which have been shown to influence the ectomycorrhizal community (Grogan et al. 2000).

Since fire is such an integral part of whitebark pine's ecology, it could be assumed that there is a mechanism for colonization of ectomycorrhizal fungi after natural fire. However, recent fire suppression has changed historical patterns, and increased fuel loading from extended fire intervals can increase fire severity, potentially resulting in stronger impacts on the mycorrhizal community (Cairney and Bastais 2007). Fire suppression also allows for advanced succession, which could shift the composition of the mycorrhizal community away from pine specialists. The general loss of whitebark pine seedlings due to rust or competition could reduce availability of the early colonizing ectomycorrhizal fungi necessary for seedling establishment after burns.

Current Knowledge of Whitebark Pine Ectomycorrhizae:

Most of the information on the ectomycorrhizal fungi associated with whitebark pine results from a few studies. A pilot study of ectomycorrhizae from roots of whitebark

pine seedlings sampled Glacier National Park showed molecular diversity but results were limited (Johnson and Kendall 1994). Perkins (2004) attempted to determine if mycorrhizae are shared between whitebark pine and the *Vaccinum* spp. often prevalent as a ground cover in whitebark pine habitats in SW Montana. Results were inconclusive, and general knowledge tells us that *Vaccinum* spp. typically hosts ericoid mycorrhizae not ectomycorrhizal fungi. However, a possible connection has been suggested for other conifers (Molina and Trappe 1982). In addition sporocarps of ectomycorrhizal fungi have been casually reported in whitebark pine forests but it is difficult to confirm an association for these reports, since other tree species may have been in close proximity (Trappe 1962, Smith and Thiers 1964, Moser et al. 1994, Moser 2002, Moser 2004).

Most recently the ectomycorrhizal fungi associated with whitebark pine were surveyed in the Greater Yellowstone Ecosystem (GYE) over 5 mountain ranges (Cripps and Mohatt 2005, Mohatt 2006, Mohatt et al. 2008). Combining both sporocarp and ectomycorrhizae data, at least 32 species of ectomycorrhizal fungi were found in pure whitebark pine forests or on seedling roots. The Boletales and Cortinariales comprised 50% of the species and most are considered host specific at some level. The suilloid fungi (Boletales) are typically restricted to hosts in the Pinaceae, and some are specific to five-needle and/or stone pines (Mohatt et al. 2008). This includes the diversity of *Suillus* and *Rhizopogon* species found to be common on regenerating whitebark pine seedlings for intact forests in the GYE.

Several taxa from the roots of whitebark pine seedlings that were determined by DNA fingerprinting using the ITS region were not observed as fruiting bodies (Mohatt et

al. 2008). This demonstrates the importance of not only sampling the aboveground fruiting bodies but also the belowground ectomycorrhizae. Some studies have shown that the abundance of representative fruiting bodies has little relationship to the belowground ectomycorrhizal community composition (Dahlberg et al. 1997, Jonsson et al. 1999a, Dahlberg 2001, Kernaghan and Harper 2001), although a study of a small aspen stand showed correlation on a small scale (Cripps 2004).

Of the ectomycorrhizal species, Mohatt et al. (2008) discovered that with whitebark pine, *Cenococcum geophilum* was the most abundant and frequent species on seedlings and was often the only ectomycorrhizae type on a root system. *Cenococcum geophilum* is a generalist found with many hosts in a diversity of habitats and studies have shown it can increase drought tolerance (Kernaghan and Harper 2001, Hasselquist et al. 2005). Since it was common on whitebark pine seedlings, it may play a critical role in the initial survival of whitebark pine seedlings, or serve as a default symbiont. Results from this study suggest that there may be a limited diversity of ectomycorrhizal fungi associated with whitebark pine trees, however additional research is needed to confirm this. Also, as the tree species declines, some species of *Suillus* and *Rhizopogon* specific to 5-needle or stone pines are likely to decline as well, and be unavailable for restoration.

Research Objectives

A large effort is being applied to the restoration of whitebark pine forests, and multiple studies are currently monitoring results. Factors that could potentially impede the progress of this research need to be examined. Planting rust-resistant seedlings after

prescribed and natural fire is one important restoration strategy. An aspect of this that has not been studied is the potential impact of fire on the community of ectomycorrhizal fungi that support whitebark pine. Previous studies have shown that fire can affect the diversity and abundance of ectomycorrhizal fungi, but results are variable, and whitebark pine forests are unique in their ecology and precipitous decline.

There were two central goals of this study: one was to examine the dynamics of whitebark pine ectomycorrhizae after fire to gain an ecological understanding, and the other was to gauge the effects of fire as a restoration treatment on mycorrhizal fungi necessary for seedling regeneration. Objectives of the ecological aspect of this research were 1) to determine the impact of fire on the ectomycorrhizal community of whitebark pine seedlings by identifying and comparing the ectomycorrhizal fungi associated with whitebark pine seedlings in a burned and adjacent unburned area, and 2) to identify ectomycorrhizal fungal species that are important to the establishment of whitebark pine after fire. The objectives of the applied aspect were to 1) determine if planted rust-resistant seedlings on the burn were mycorrhizal, 2) compare the ectomycorrhizal communities of planted rust-resistant seedlings inside burned areas with those naturally regenerating within the burn and those that are naturally regenerating in mature forests, 3) to identify ectomycorrhizal fungi that have possible potential as an inoculum source for use in nurseries. This research will contribute to the understanding of the ecology of whitebark pine forests and their return and succession after fire. This information will help managers trying to restore these forests by giving them one more measure of forest health.

CHAPTER 2

A COMPARISON OF ECTOMYCORRHIZAL FUNGI ON NATURAL AND PLANTED WHITEBARK PINE SEEDLINGS IN BURNED AND UNBURNED SOILS

Introduction

Whitebark pine (*Pinus albicaulis* Dougl. ex Hook.) is a keystone species in the Greater Yellowstone Ecosystem (GYE) as it fosters biodiversity in harsh, high elevation environments, provides a major component of grizzly bear diet (pine nuts), and helps maintain watershed health (Tomback et al. 2001, Primack 2002, Schwandt 2006). Whitebark pine is the only North American species of stone pine, which are all characterized by large indehiscent cones, an avian dispersal agent (Clark's nutcracker for whitebark pine), and five-needle fasicles. Whitebark pine has experienced extreme losses, 40-90% throughout its range, due to white pine blister rust, mountain pine beetles, climate change, and fire suppression (Keane and Arno 1993, Tomback and Kendall 2001). Due to the importance of whitebark pine in the upper subalpine ecosystem where it exists, intensive restoration efforts have been implemented, including the planting of over 200,000 rust resistant whitebark pine seedlings since 1989 (Keane et al. 2001).

Forest managers are testing the reintroduction of fire as a way to restore these forests (Keane and Arno 2001). Fires have historically been a key aspect in whitebark pine forest ecology. Burning eliminates the less fire tolerant competitors of whitebark pine (Engelmann spruce and subalpine fir), which will replace whitebark pine locally if unchecked due to their shade tolerance (Arno 2001, Schwandt 2006). Fires also produce

more open spaces for Clark's nutcrackers to cache whitebark pine seeds with enough light for them to grow (Tomback et al. 2001). Today forest managers want to re-establish fire in whitebark pine ecology and use it to contend with modern problems. Managers hope burning will reduce the number of rust infected trees, and rust resistant seedlings can be planted in the openings created by the fire which increases the potential for naturally resistant trees to develop (Kendall and Keane 2001). In addition, burning out competitors of whitebark pine will reduce stress from competition and reduce the number of mature trees, making forests less prone to beetle attacks, as beetles preferentially infect large older trees (Arno 1986, Kendall and Keane 2001).

An important consideration is how the reintroduction of fire will impact these advanced successional and fire-suppressed whitebark pine forests. "While there is a wealth of literature on how fire affects above-ground vegetation, its impacts on the below-ground component of forest ecosystems is poorly understood" (Cairney and Bastias 2007).

The ectomycorrhizal fungi associated with whitebark pine are a critical component of the below-ground microbial community. Pines are obligate mutualists with ectomycorrhizal fungi and normal growth cannot occur without them in nature (Smith and Read 1997, Read 1998). In exchange for a source of carbon the fungal partner in an ectomycorrhizal relationship can confer many different benefits onto the host tree including increased phosphorus and nitrogen uptake, increased water potential, direct protection from root pathogens, and an overall increase in health, depending on the fungus involved (Morin et al. 1999, Nardini et al. 2000, Landhausser et al. 2002).

Ectomycorrhizal fungi also exhibit host specificity and follow successional patterns. Ectomycorrhizal fungi can be generalists with a wide host range or be restricted to one host species (Bruns et al 2002, Mohatt et al. 2008). In succession, the general thought is that ectomycorrhizae of generalist fungi are typically on young seedlings and are followed by more host-restricted types as forests age (Trappe 1977, Molina and Trappe 1982, Allen 1992). However, suilloid fungi specific for pines are considered both early and late colonizers.

Recent research has discovered many of the ectomycorrhizal fungi associated with whitebark pine trees in the Greater Yellowstone Ecosystem (GYE) where abundant whitebark pine forests still exist (Mohatt et al. 2008). At present 32 species are recorded with this tree species for five mountain ranges. However, nothing is known of how fire impacts the communities of ectomycorrhizal fungi associated with whitebark pine trees. We also do not know if nursery seedlings planted in a burn are colonized by ectomycorrhizal fungi in a timely manner, and if the fungi involved are the same as those that colonize naturally establishing seedlings. While multiple studies have examined the effect of fire on the ectomycorrhizae of other trees including pines, results have often appeared inconsistent or contradictory (Visser 1995, Miller et al. 1998, Dahlberg et al. 2001, Smith et al. 2004, Tuininga and Dighton 2004, Hart et al. 2005a, Smith et al 2005). Some studies report no changes in the ectomycorrhizae after fire (Korb et al. 2004), while others have found altered ectomycorrhizal communities (Grogan et al. 2000), decreased species richness (Smith et al. 2005), and altered relative abundance (Jonsson et al. 1999b). Upon review, it appears that many of these discrepancies can be explained by

variations in the intensity/severity of the burn, the amount of time since the burn, and the frequency of burning (Neary et al. 1999, Cairney and Bastias 2007). Even with these few predictors the impacts of fire on forest systems are still considered site specific and all fires behave differently (Cairney and Bastias 2007).

There were two central purposes to this study; one was to examine the dynamics of whitebark pine ectomycorrhizae after fire to gain an ecological understanding of the process and the other was to gauge the effectiveness of fire as a restoration treatment as it concerns mycorrhizal colonization. Objectives of the ecological aspect of this research were 1) to determine the impact of fire on the ectomycorrhizal community of whitebark pine seedlings by identifying the ectomycorrhizal fungi associated with seedlings in a burned area and comparing them to seedlings in unburned areas, and 2) to identify ectomycorrhizal fungal species involved in the establishment of whitebark pine after fire. The objectives of the applied aspect were to 1) determine if planted rust resistant seedlings on the burn were colonized by fungi mycorrhizal, and 2) compare the ectomycorrhizal communities of planted rust resistant seedlings inside burned areas with those naturally regenerating within the burn and those that are naturally regenerating in mature forests. The study was conducted in the Gallatin National Forest (MT) on an area burned by the Fridley Fire in 2001 and on an adjacent unburned whitebark pine forest.

This research will contribute to the understanding of the ecology of whitebark pine forests and their return after fire. Results will supply one more measure of forest health for managers trying to restore these forests.

Materials and Methods

Site Description:

The study site was located at the junction of West Pine Creek and North Dry Divide Trail within the Gallatin National Forest in Gallatin/Park County, Montana (N 45°28.603'; W 110°48.820'). The sampling area consisted of an almost pure whitebark pine forest, a large portion of which burned in the 2001 Fridley Fire. Subsequently, 20,880 whitebark pine seedlings were planted in the burned area in the summer of 2002 (GNF Archives 218-01-067).

The study area was located between 2600 and 2750 meters in elevation with a flat aspect (Figure 2.1). The average annual precipitation for the research site is between 51 and 89 cm. Annual precipitation in the years between the planting of whitebark pine and initiation of the study at the closest SNOTEL monitoring site (Lick Creek) were 58 cm in 2001, 63 cm in 2002, 62 cm in 2003, 70 cm in 2004, 70 cm in 2005, and 83.1 cm in 2006. For July and August of 2006, the months that samples were obtained, precipitation was 4.1 cm and 2.8 cm respectively (NRCS website). The soil composition is a mollic cryoboralf with volcanic substratum and has a typical profile of very gravelly loam (Soil Survey Staff 2008). For the unburned area, organic matter comprised 4.5% of soil material less than two millimeters is size and the average soil pH for the sampling area was six. Mean annual air temperature is between 2.2°C and 3.9°C. Soil temperature was measured four times at a depth of four centimeters during the sampling period in burned and unburned areas (Table 2.1). On each date, soil temperature was sampled 18 times, along each transect at 0, 100, and 200 meters. The mean soil temperature was calculated

for each day and a t-test in Program R was used to determine any significant differences (R Development Core Team 2009). For each sample day, soil temperatures were significantly higher in the burned area than the unburned area. See Appendix A for more detailed soil temperature data.

Table 2.1: Mean soil temperatures in °C on four sampling days for burned and unburned soil and significance values for comparisons.

Date	Unburned Mean	Burned Mean	Degrees Freedom	P-value
7/12/2006	14.7°C	20.2°C	16	6.47E-07
7/17/2006	16.3°C	24.3°C	16	1.67E-07
7/29/2006	17.7°C	25.1°C	16	6.19E-08
8/08/2006	15.4°C	23.6°C	16	6.56E-04

The intact part of the forest consisted of mature whitebark pine trees with an open understory. Regeneration of multiple-aged whitebark pine trees occurs throughout the stand. A small number of subalpine fir was intermixed with the whitebark pine trees in some areas. The ground cover consisted of thick mats of *Vaccinium scoparium* (grouse whortleberry). Minimal damage due to white pine blister rust was also observed on a few whitebark pine trees. The fire history for the site is unknown but some areas show fire scars from burns in the 1880's and the 1940's (Northern Rockies National Incident Management Team 2001).

In 2001, the lightning-ignited Fridley Fire burned 10,700 hectares of the Gallatin National Forest including a section of the whitebark pine stand described above. The West Pine Creek trail was rerouted after the fire and currently follows a portion of the northeastern burn boundary and provides access to the area.

The fire had a high burn intensity and moderate/high burn severity within the burned sampling area (Fridley Fire BAER Team 2001). Burn intensity was measured as

the effect of the fire on the vegetation of the area. High burn intensity is described as producing intense heat and blackening of at least 75% of all trees with all ground vegetation being consumed. Within the burned sampling area 100% of the trees were killed.

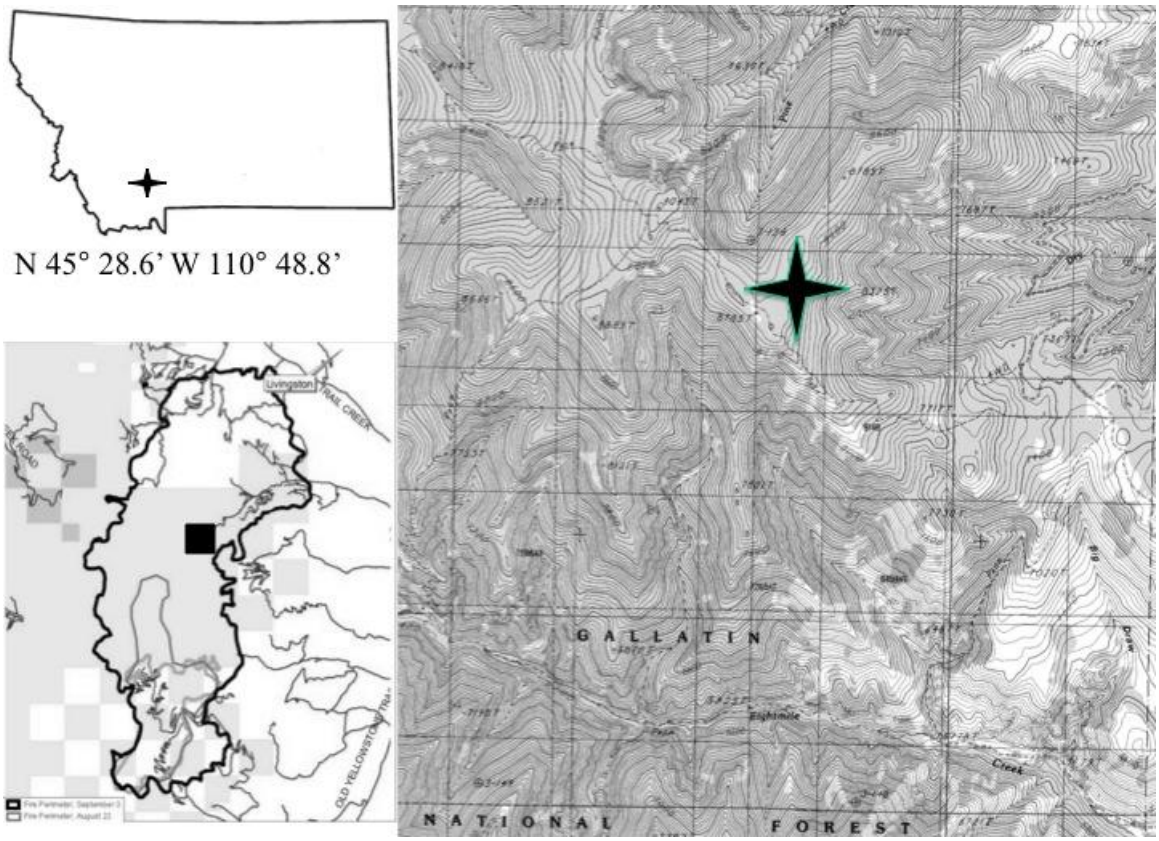


Figure 2.1: Location of Fridley Burn within Montana, perimeter of Fridley Burn (2001) with study site marked. Topographic map (USGS Big Draw) with research sampling area marked. N 45^{o} 28.6' W 110^{o} 48.8', T4S R7E S24.

Burn severity was measured as the effect of fire on the ecosystem, mainly soils. Medium/high burn severity within the research area was evidenced by complete consumption of the litter and duff, a change in the color of the mineral soil, a hydrophobicity of 2.5 to 5 cm in depth, and lethal temperatures extending down 5 to 10

cm. The area is currently covered with standing dead and many downed burned trees (primarily whitebark pine) and the ground area is completely exposed in the absence of canopy cover. Five years after the burn, the understory composes dense *Chamerion angustifolium* (L.) Holub. (= *Epilobium angustifolium* L.) (fireweed) and various grasses.

The study area consisted of two sub-areas: one consisting of an unburned mature whitebark pine forest along West Pine Creek trail, and a 28-hectare burn area. The burn area was replanted with rust resistant whitebark pine seedlings in 2002 adjacent to the North Dry Divide Trail (Figure 2.2). These 28 hectares were replanted with 20,880 two-year-old whitebark pine seedlings grown at the USDA Coeur D'Alene Forest Service Nursery to enhance regeneration (GNF Archives 218-01-067). Whitebark pine is also regenerating naturally within the burned area. The natural seedlings can be differentiated from planted nursery stock because they are smaller, the root system is less extensive and not in a conetainer shape, and seedlings are often in clusters. The mycorrhizal assessment was done in 2006, five years after the fire and four years after seedlings were planted.

Sampling Design:

Six transects, three in the burned and three in the unburned area (Figure 2.3) were established within the research site near West Pine Creek Trail (TRL 139) and North Dry Divide Trail (TRL 135) in the Gallatin National Forest. Each 200 meter transect initiated at the trail and extended perpendicular to the trail (Figure 2.2). Transects were placed 200 meters apart and ran parallel to each other within each sub-area. The slope of transect areas were flat and aspect was northeast.

Roots of seedlings were sampled twice, once at the beginning of July (1st-14th) and again at the beginning of August (July 29th-Aug. 14th) in 2006. Transects in burned and unburned areas were sampled in alternating order to reduce any bias due to date. Root samples were taken from whitebark pine seedlings along transects approximately every 20 meters for a total of ten collections per transect and thirty each in burned and unburned areas per sampling period. Samples for the second period were taken from different seedlings than those used in the first sample. Seedlings were defined as over three but less than ten years of age as estimated by the number of whorls.

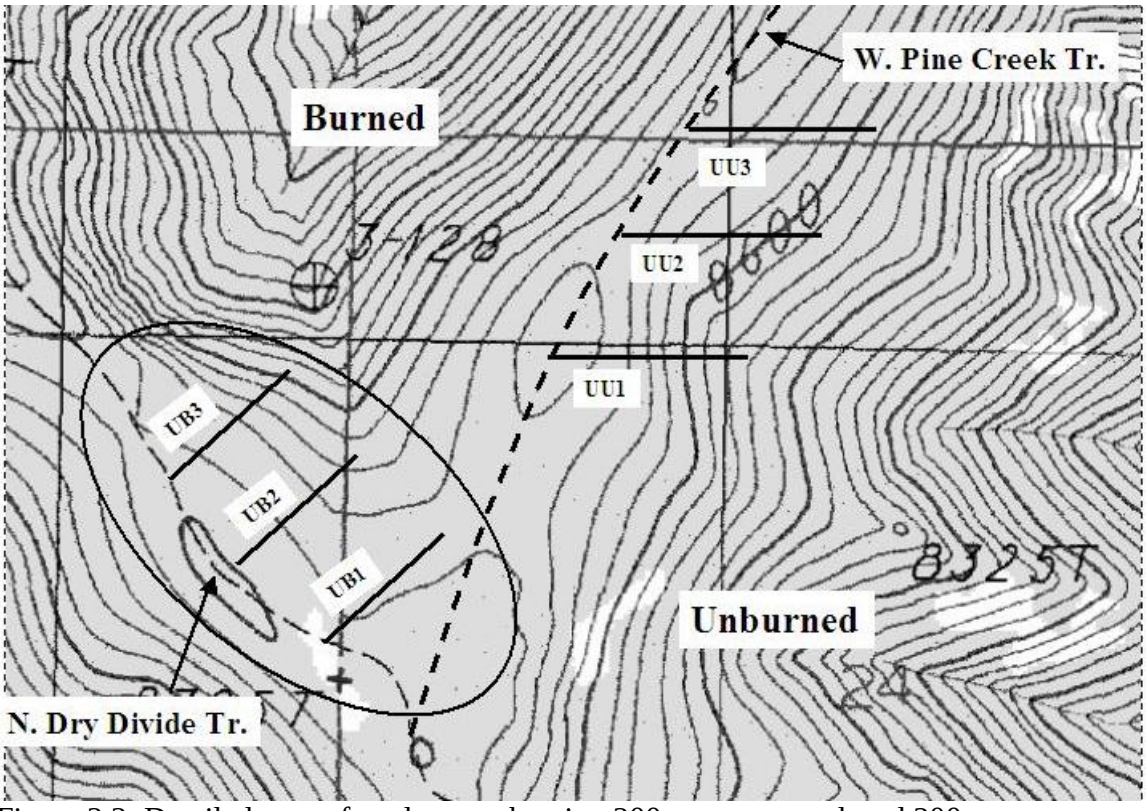


Figure 2.2: Detailed map of study area showing 200 m transects placed 200 meters apart. To the left of West Pine Creek trail is the Fridley Burn (Gallatin National Forest, MT) and to the right is unburned mature whitebark pine forest. The circle designates the area where rust-resistant whitebark pine seedlings were planted in 2001. UU=unburned transects, UB=burned transects.

Figure 2.3: Study sites on and adjacent to the Fridley Burn, Gallatin National Forest, MT. A) Unburned whitebark pine forest located to the West of West Pine Creek Trail B) Burned whitebark pine forest to the East of the trail. Images show late summer snowpack

Seedlings closest to the transect at a sample location were selected for sampling. Seedlings were all within five meters of the transect sample point. Naturally regenerating seedlings within the burned area were limited in number but at least ten were sampled per period. The total sample comprised root samples from sixty seedlings that were planted within the burn, sixty seedlings regenerating naturally in the unburned area, and twenty-four seedlings regenerating naturally in the burn.

It was necessary to develop a non-destructive sampling method due to Forest Service constraints. Each seedling was carefully dug up in a manner that kept a majority of the root system intact. Sections were taken from the top, middle, and lower portion of each root system and seedlings were then replanted. Roots obviously infected with ECM were preferentially sampled from each root zones for all three treatments. Root samples were removed with clippers and placed in containers filled with de-ionized water. Samples were then transported to the laboratory in a cooler, and typically processed within 24 hours to a few days depending on the number of samples.

A few seedlings were collected for a comparison of root systems (Figure 2.4).

Figure 2.4: Examples showing differing root and shoot sizes of whitebark pine seedlings sampled. From upper right clockwise: natural unburned, natural burned, planted burned.

Ectomycorrhizal Morphotyping:

For the non-destructive sampling technique, 25 to 40 cm of root were removed in the field from top, middle, and lower sections, leaving the rest of the root system intact (Brundrett et al. 1996). This allowed 100% of the root tips in a sample to be analyzed. Root sections were washed and placed in petri plates of deionized water for examination with a dissecting microscope. Ectomycorrhizal root tips were recognized by the presence of a mantle, extramatricular hyphae or rhizomorphs for some, and the dichotomous branching typical of pines. For each sample, ectomycorrhizal tips were then separated into morphotypes based on branching patterns, mantle color, and hyphal and rhizomorph characters (Agerer 1987-2006). Ectomycorrhizal tips suspected of being *Cenococcum*

geophilum were examined with a microscope for the distinctive star-shaped mantle pattern so that molecular identification would be unnecessary. Root tips of each morphotype were counted to determine abundances within each sample. Non-mycorrhizal and non-viable ectomycorrhizal root tips were also counted; the latter were defined by a dark color and desiccated appearance due to low turgor. Each morphotype was sorted according to general characteristics.

A representative sample of each ectomycorrhizal morphotype from each seedling was stored separately in a 1.5 mL centrifuge tube in 1 mL of 2% CTAB and placed in a –20^{o}C freezer for subsequent molecular work. If more than 15 root tips of a morphotype were available, a voucher collection was placed in deionized water and saved at –20^{o}C.

DNA Extraction:

DNA was extracted from ectomycorrhizal morphotypes using a modified version of the Mo Bio Powersoil™ DNA isolation protocol. This protocol was selected in order to limit inhibitory compounds such as humic acid, which can be difficult to remove completely from mycorrhizal samples. Samples were removed from 2% CTAB and placed in a 2 ml impact-resistant screw top tube containing garnet and a 6.5 mm ceramic bead. Next 740 µl of Powerbead Solution® and 60 µl of Solution I® were added to the sample. To lyse cells, samples were placed in FastPrep® Instrument (Qbiogene Inc., Irvine, CA) for 45 seconds at a setting of 6.0. The samples were then cooled at room temperature for five minutes. After cooling, samples were centrifuged at room temperature for 10 minutes at 13,000 g. As much liquid as possible (400-500 µl) was then removed from the sample tube and transferred to a fresh 2.0 ml tube. In the new tube, 250

µl of Solution 2® was added to the supernatant, which was then vortexed for 5 seconds, and placed in a refrigerator at 4ºC for 5 minutes. After cooling, samples were spun for 1 minute at 13,000 g. Next, 600 µl of the supernatant was transferred to a fresh 2 ml tube, making sure to avoid the pellet at the bottom. To the supernatant, 600 µl of DNA Binding Matrix Solution was added and mixed for 5 minutes on a horizontal shaker. The samples were flash-spun for 12 seconds and the supernatant discarded. The remaining pellet was re-suspended with 600 µl of ice cold 80% ethanol and washed for 2 minutes on the horizontal shaker. After washing, samples were flash spun for 12 seconds and the ethanol was discarded, and then washed with ethanol again. After the second wash, flash spinning for 12 seconds re-suspended the binding matrix and excess ethanol was removed via pipetting. To remove any possible ethanol left in a sample, each was placed in the speed - vac for 10 minutes at medium heat setting. To the dry matrix, 100 µl of sterile water was used to re-suspend the sample. The samples were then placed in a 55^{o}C water bath for 5 minutes. In order to pellet out the binding matrix, samples were spun at 13,000 *g* for 1 minute. Making sure to avoid the pellet, 65 µl of the supernatant was removed by pipette and placed into a new 2 ml tube. To pellet out any remaining DNA binding matrix, the samples were spun again at 13,000 *g* for 1 minute. Of the new supernatant, 50 µl was pipetted off and placed into a fresh 1.5 ml tube and stored at –20^{o}C.

PCR Amplification and Sequencing:

In order to relate the ectomycorrhizal morphotypes to a taxonomic group, the ITS (internal transcribed spacer) region of fungal ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR). Amplification was accomplished by mixing 1µl of

DNA extracted from a morphotype with 1 µl of the fungal-specific ITS1-F primer (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993), 1 µl of reverse primer, either ITS4 (TCCTCCGCTTATTGATATGC) (White et al 1990) or Lr21 (ACTTCAAGCGTTTCCCTTT) (Tedersoo 2007), 14 µl of Jumpstart ReadyMix (product of Sigma™), containing 20 mM Tris-HCL (pH 8.3), 100 mM KCL, 4 mM $MgCl_2$, 0.002% gelatin, 0.4 mM each dNTP (dATP, dGTP, dCTP, TTP), inert dye, stabilizers, 0.06 unit/µl Taq DNA Polymerase, Jumpstart Taq antibody, and 8 µl of DNase-free water to a final volume of 25 µl. The morphotypic characters of the ectomycorrhizae helped determine which reverse primer was used initially. If a thin-mantle or other feature suggested the fungus belonged to the Ascomycota, the universal primer ITS4 was used. If characters such as clamps or a particular mantle type suggested it belonged to the Basidiomycota, the Basidiomycota-specific primer TR-21 was used initially. If amplification failed with the more specific TR-21 primer, the sample was re-amplified with the general fungal reverse primer ITS4. If the general fungal primer failed to produce a single band during gel electrophoresis, the alternate Basidiomycota primer ITS4-B was used.

Samples were amplified in an Eppendorf Mastercycler Gradient thermocycler (Brinkman Instruments, Westbury, NY, USA) using a protocol modified from Gardes and Bruns (1993). The thermocycler protocol was five minutes at 95°C, then 35 cycles of 1 minute at 95°C, thirty seconds at 55°C, and 1 minute at 72°C. After 35 cycles, the product was placed at 72°C for 10 minutes. Each time samples were amplified, a positive control was run using a sample that amplified consistently to ensure that the amplification

reaction had occurred. In addition to the positive control, a negative control was run with all amplification reagents by replacing the sample DNA with DNase-free water. This was done to assure that any amplified products were not due to contamination.

After the samples were run through the thermocycler protocol, they were visually examined for amplification by gel electrophoresis and visualization with UV light. Five μL of each amplified sample were added to a 1.5% agarose gel and run at 74 amps for one hour. At the end of the hour, the samples were visualized in a UV light box and photographed. The samples were compared to a 1 Kb molecular ladder to make sure that they were of the approximate correct size for the ITS region for fungi (600-700bp).

If the amplification process was successful, the remaining sample PCR product was cleaned using Montage™ PCR Centrifugal Filter Devices (Millipore Corp., Billerica, MA, USA) according to the manufacturer's protocol.

Sequencing reactions were carried out by the University of California at Berkeley DNA Sequencing Facility using the ITS1-F primer (Berkeley, CA). Sequences were manually cleaned using Finch TV (Geospiza website) and compared to the NCBI GenBank database using BLAST search and described as the closest taxonomic match (Altschul et al. 1990, Altschul et al. 1997). The percent match required for confidence in the taxonomic grouping suggested by BLAST is highly dependent on the particular fungal taxon and was considered on a case-by-case basis, typically 96-97%.

Statistical Analysis:

After the ectomycorrhizal fungal taxa were identified by molecular techniques and morphotyping, data were analyzed quantitatively and statistically. Data from the two different sampling times were pooled after it was determined that there were no statistical differences between them. The average number of fungal species per seedling was calculated for the three mycorrhizal communities, and Analysis of Variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) were used to compare species richness among the three seedlings groups using Program R (R Development Core Team 2009). The relative abundance and frequency for the most encountered ectomycorrhizal fungal taxa were also determined and summed for importance values (Horton and Bruns 2001, Cripps 2004). Shannon's and Simpson's diversity indices compared diversity among groups (done in Program R). Kruskal's Non-metric Multidimensional Scaling (NMDS) was used to map Bray-Curtis dissimilarity values for the ectomycorrhizal communities and to visually examine the structure of the ECM communities based on whitebark pine seedling groupings. NMDS was performed and graphed for the three whitebark pine seedling groups: natural unburned, natural burned and planted burned using R. Pair-wise multi-response permutation procedures were also used to evaluate group effects on the abundances of the eight most common taxa combined using R.

In addition to the NMDS plots, a Principal Components Analysis of the log-transformed abundances was also plotted for the ectomycorrhizal fungal communities. The plot provides visual representation of the ectomycorrhizal fungal communities and includes loading vectors for taxa that have an absolute value loading score of at least 0.1.

This allows for easy interpretation of which taxa are driving sample distances. Principal components analysis (PCA) was used to examine the structure of ectomycorrhizal fungal communities based on whitebark pine seedling groupings.

Results

A total 21,971 root tips were examined from 144 whitebark pine seedlings (Table 2.2). The root tips of whitebark pine seedlings planted in the burn were 96% mycorrhizal, 65% of the tips were viable and 32% were nonviable. Whitebark pine seedlings naturally regenerating in the unburned mature forests were 99% mycorrhizal, 34% viable and 65% nonviable. Whitebark pine seedlings that were naturally regenerating inside the burned area were 91% mycorrhizal, 46% viable and 45% non-viable.

Table 2.2: Viable and non-viable ectomycorrhizae, and non-ectomycorrhizal root tips on whitebark pine seedlings for three groups.

Seedling Group	N	ECM Tips	Non ECM Root Tips	Total Tips	% Mycorrhizal
Natural Unburned	60	11209	42	11251	99.6
Natural Burned	24	1997	203	2200	90.8
Planted Burned	60	8215	305	8520	96.4

Examination of the ectomycorrhizal root tips yielded 22 distinct taxa, all of which, with the exception of *Cenococcum geophilum*, were identified by DNA fingerprint analysis of the ITS region. The six most common morphotypes are shown in Figure 2.5. For identification of these taxa, the ITS region was sequenced for 136 samples and 67 sequences provided information. Of the sequenced taxa, 27 were from natural seedlings in the unburned forest, 15 from natural seedlings in the burned area, and 25 from seedlings planted on the burn. These sequences were used to determine 21

unique taxa by closest match with NCBI blast search (Altschul et al. 1990, Altschul et al.1997) search (Table 2.3).

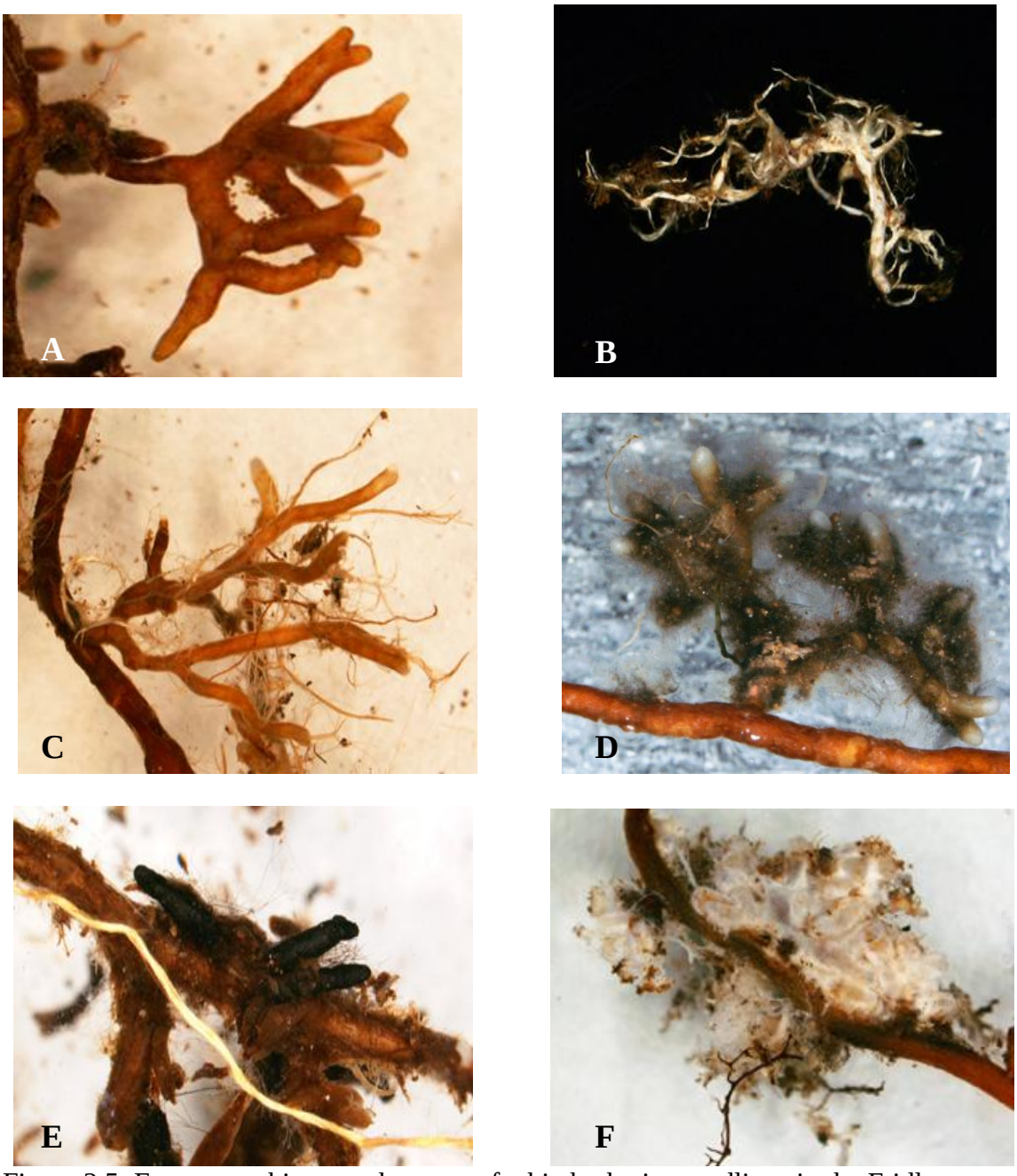


Figure 2.5: Ectomycorrhizae on the roots of whitebark pine seedlings in the Fridley research area. Morphotypes: A. *Wilcoxina* spp., B. *Piloderma byssinum*, C. *Amphinema byssoides*, D. *Pseudotomentella nigra*, E. *Cenococcum geophilum*, F. *Rhizopogon* spp.

The taxa identified as in the *Rhizopogon subbadius*/*salebrosus* clade and *Rhizopogon roseolus* clade each contained multiple possible species. The species within these clades show strong similarity in the ITS region that was used for identification. Rather than attempt to identify each of these taxa to species and risk misidentification which could result incorrect quantification of richness and diversity, each was placed in clades as done by Grubisha et al. (2002) in their examination of *Rhizopogon* species.

Neonectria radicicola (= *Cylindrocladium* sp.) is not ectomycorrhizal but was included in the table because it is a pathogen of whitebark pine roots.

Table 2.3: Taxa matches for identification of ectomycorrhizal fungi on whitebark pine seedlings using NCBI Blast.

Study name	Best match	% Identity	Overlap (bp)
Amphinema byssoides	Amphinema byssoides (AY838271)	97%	581/593
Coltricia sp.	Coltricia perennis (DQ234560)	94%	745/785
Cortinarius near sp. brunneus	Cortinarius brunneus (AF430287)	96%	672/693
Phialocephala fortinii	Phialocephala fortinii (AY394921)	99%	531/533
Piloderma byssinum	Piloderma byssinum (DQ365683)	97%	422/432
Pseudotomentella nigra	Pseudotomentella nigra (UDB000281)	96%	531/549
Rhizopogon roseolus clade	Rhizopogon roseolus (UDB001619)	97%	705/726
Rhizopogon subbadius/salebrosus clade	Rhizopogon subbadius (AF377152)	98%	763/774
Russula near sp. turci	Russula turci (EF530935)	96%	688/714
Sebacina species	Sebacinaceae (DQ273405)	94%	476/503
Suillus variegatus	Suillus variegatus (AY898622)	98%	669/682
Suillus umbonatus	Suillus umbonatus (L54115)	99%	592/593
Suillus placidus/subalpinus	Suillus placidus (DQ407265)	96%	627/647
Thelephora sp.	Thelephora terrestris (FJ532478)	93%	568/609
Tomentella sp.	Tomentella subalcina (UDB002972)	91%	744/809
Wilcoxina mikolae	Wilcoxina mikolae (DQ069000)	98%	579/587
Wilcoxina rehmii	Wilcoxina rehmii (AF266708)	98%	510/520
Wilcoxina sp. 1 cf. mikolae	uncultured Wilcoxina (DQ320129)	97%	561/575
Species 1	uncultured fungus (EU292455)	97%	743/761
Species 2	uncultured Agaricales (FJ475683)	98%	645/654
Species 3	uncultured ectomycorrhiza (DQ481981)	97%	617/634
Neonectria radicicola (NOT ECM)	Neonectria radicicola (AJ875336)	99%	528/531

After identification of taxa, the species richness per seedling was calculated (Table 2.4). The mean richness of ectomycorrhizal fungal species was 1.6 per planted seedling inside the burn, 1.6 per naturally regenerating seedlings inside the burn, and 2.2

for seedlings regenerating naturally within the unburned forest. A single factor ANOVA followed by a Tukey's HSD test found seedlings naturally regenerating in the unburned forest to be significantly richer than both planted and naturally regenerating trees in the burn (p-value=0.0002).

Table 2.4: Taxa richness of ectomycorrhizal fungi on whitebark pine seedlings by group and test score for single factor ANOVA.

	Richness (Standard Deviation)	
Natural Unburned	2.17 ± (0.64) A	
Natural Burned	1.58 ± (0.83) B	
Planted Burned	1.58 ± (0.77) B	p-value < 0.0002

Table 2.5: Relative frequency and abundance most common ectomycorrhizal taxa on whitebark pine seedlings.

Taxa	Natural Unburned		Natural Burned		Planted Burned	
	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance
Amphinema byssiodes (Pers.) J. Erikss.	0.02	0.03	0.05	0.11	0.23	0.13
Cenococcum geophilum Fr.	0.42	0.37	0.27	0.08	0.10	0.02
Cortinarius sp.	0.02	0.01	0.00	0.00	0.00	0.00
Piloderma byssinum (P. Karst.) Jülich	0.28	0.25	0.03	0.00	0.01	0.01
Pseudotomentella nigra (Höhn. & Litsch.) Svrček	0.02	0.01	0.19	0.18	0.21	0.44
Rhizopogon roseolus (Corda) Th. Fr.	0.05	0.09	0.03	0.02	0.01	0.00
Rhizopogon subbadius A.H. Sm.	0.03	0.06	0.08	0.07	0.02	0.01
Unknown Rhizopogons	0.02	0.01	0.03	0.02	0.01	0.01
Suillus placidus/subalpinus (Bonord.) Singer	0.01	0.00	0.00	0.00	0.00	0.00
Suillus umbonatus E.A. Dick & Snell	0.00	0.00	0.00	0.00	0.01	0.04
Suillus variegatus (Sw.) Kuntze	0.02	0.01	0.00	0.00	0.00	0.00
Unknown Suilloids	0.09	0.11	0.00	0.00	0.03	0.01
Thelephoroid spp.	0.00	0.00	0.00	0.00	0.06	0.05
Wilcoxina mikolae (C.S. Yang & H.E. Wilcox) C.S. Yang & Korf	0.00	0.00	0.05	0.05	0.03	0.03
Wilcoxina rehmii C.S. Yang & Korf	0.00	0.00	0.00	0.00	0.01	0.01
Wilcoxina spp.	0.03	0.02	0.24	0.37	0.19	0.19

The fungal communities on different seedling groups were compared in terms of importance values, relative frequencies, and relative abundances for the most common ectomycorrhizal fungal species (Figure 2.6). While many of these ectomycorrhizal taxa occur on all seedling groups, at least in low levels, changes in importance values between natural seedlings on the unburned area and planted seedlings on the burned area are so extreme that it can be considered a near complete species shift. The importance values for fungal taxa on naturally regenerating seedlings inside the burned area fall between the values of the other two seedling groups (Figure 2.7).

Taxa that have the highest importance value in the unburned area are *Cenococcum geophilum*, *Piloderma byssinum*, and the suilloid fungi taken as a group. The importance value of *Cenococcum geophilum* (0.78) on naturally regenerating whitebark pine seedlings in the unburned area is two and five times higher than those for natural (0.34) and planted (0.13) seedlings inside the burned area, respectively. Seedlings in the unburned area also had a higher relative frequency and abundance of *Piloderma byssinum* than either group inside the burned area where it was negligible. For this fungus, seedlings in the unburned area had an importance value of 0.53, which is 18 times higher than for either group of seedlings in the burned area. The suilloid fungi as a group (genera *Rhizopogon* and *Suillus*) had a higher importance value in the unburned area than on the burned area.

Relative Frequency Relative Abundance

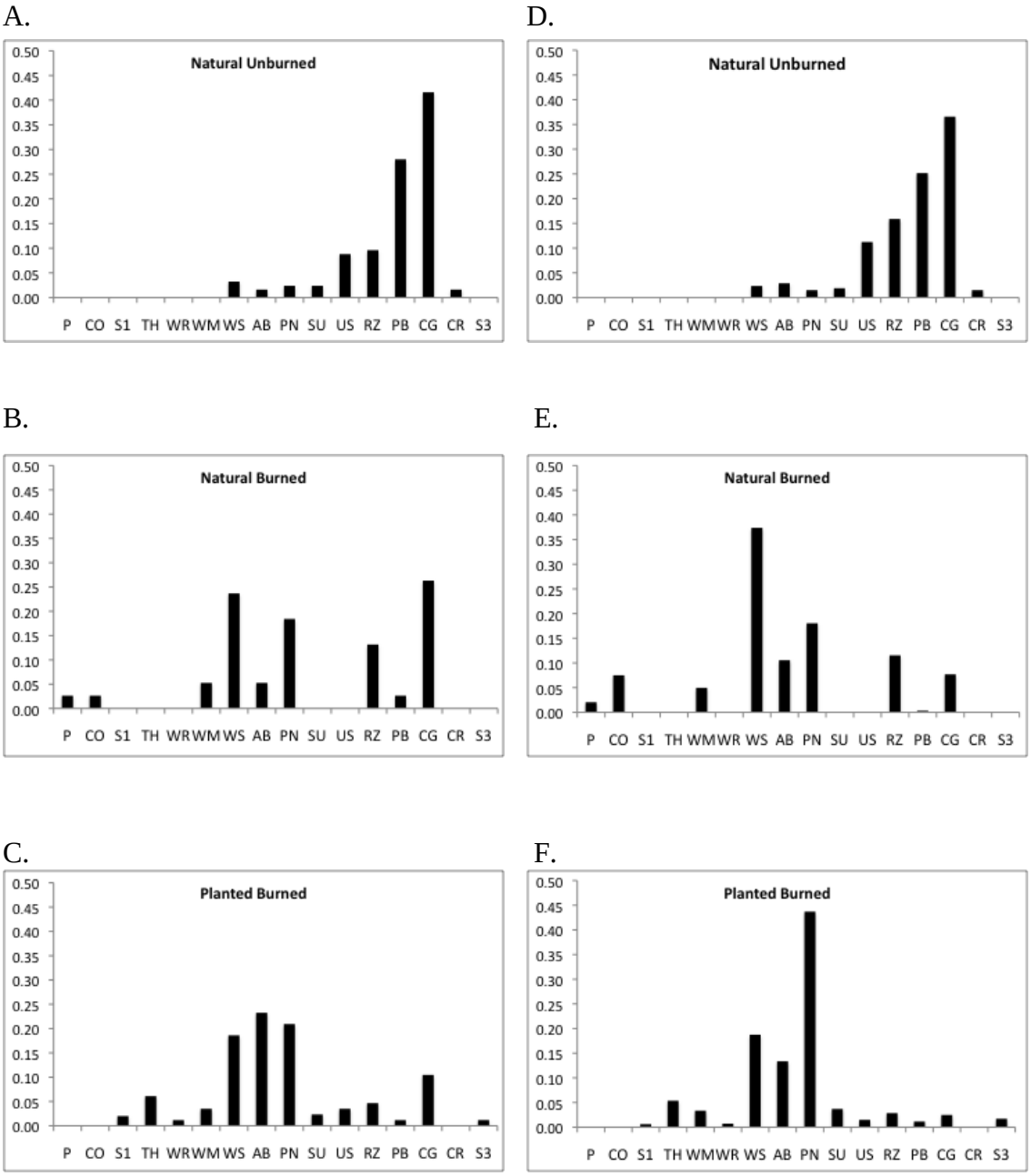


Figure 2.6: Relative frequency (A-C) and relative abundance (D-F) of ectomycorrhizal taxa on whitebark pine. Abbreviations: P-*Phialocephala fortinii*, S1- Species 1, CO-*Coltricia* sp., TH-Thelephoroid spp., WM- *Wilcoxina mikolae*, WR- *Wilcoxina rhemii*, WS- *Wilcoxina* spp., AB- *Amphinema byssoides*, PN- *Pseudotomentella nigra*, SU-*Suillus* spp., US- Unknown Suilloids, RZ- *Rhizopogon* spp., PB- *Piloderma byssinum*, CG- *Cenococcum geophilum*, CR- *Cortinarius* spp., S3- Species 3.

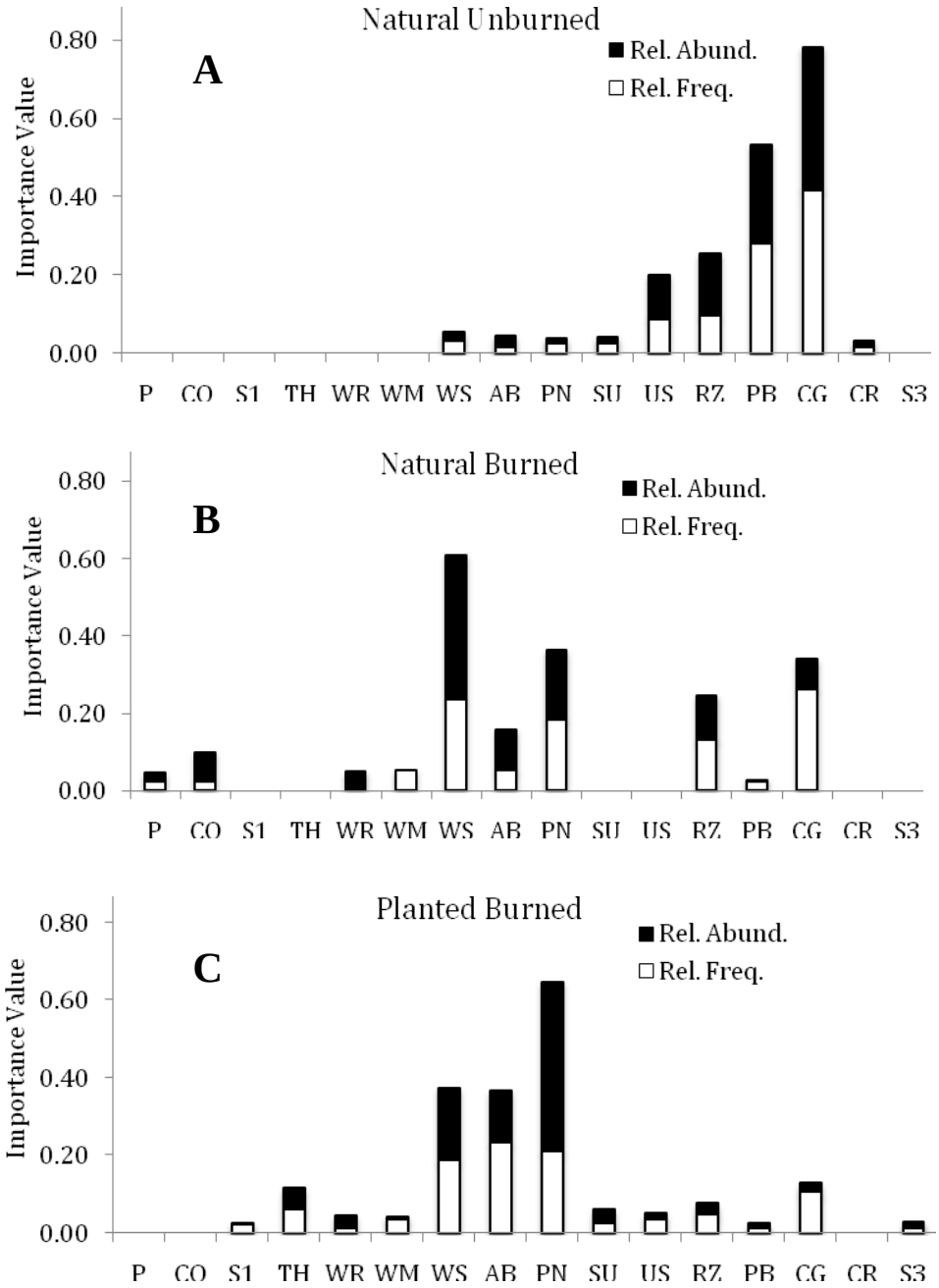


Figure 2.7: Importance values for ten most common taxa of ectomycorrhizal fungi on whitebark pine seedlings. A. regenerating naturally in the unburned forest, B. regenerating naturally in burned area, C. planted in burned area. Abbreviations: P- *Phialocephala fortinii*, S1- Species 1, CO- *Coltricia* sp., TH-Thelephoroid spp., WM- *Wilcoxina mikolae*, WR- *Wilcoxina rhemii*, WS- *Wilcoxina* spp., AB- *Amphinema byssoides*, PN- *Pseudotomentella nigra*, SU- *Suillus* spp., US- Unknown suilloids, RZ - *Rhizopogon* spp., PB- *Piloderma byssinum*, CG- *Cenococcum geophilum*, CR- *Cortinarius* spp., S3- Species 3.

In the burned area *Pseudotomentella nigra*, *Amphinema byssoides*, and *Wilcoxina* species are dominant. The importance value of *P. nigra* is about two to 16 times higher on seedlings in the burn compared to those on the unburned area. *Amphinema byssoides* has importance values of 0.16 on natural and 0.37 on planted seedlings in the burn, which are three and seven times higher than the 0.05 for natural seedlings in the unburned area. *Wilcoxina* species have an importance value about ten times higher on natural (0.7) and planted (0.5) seedlings in the burn respectively, compared to those growing in the unburned (0.06) area.

While these dominant taxa occur on all groups of whitebark pine seedlings, other fungi are only reported on certain groups of seedlings. For example, Thelephoroid spp. were only on planted seedlings and *Cortinarius* species only on naturally regenerating seedlings inside the unburned area. However, the importance values of these taxa, 0.11 and 0.03 show they are not the most dominant for their particular seedling groups.

The dissimilarities in taxa among seedling groups are supported by differences in mean abundance per seedling (Table 2.6). A single factor Analysis of Variance (ANOVA) was used to determine if any significant differences existed among groups in mean abundances per seedling for the most common taxa. When a significant difference was found, the ANOVA was followed by a Tukey's Honest Significant Difference (HSD) to further analyze where differences occurred among groups.

Table 2.6: Relevé table of mean abundance for ten most common taxa of ectomycorrhizae. Letters indicate statistical significance by ANOVA followed by HSD tests.

Taxa	Natural Unburned N = 60	Natural Burned N = 24	Planted Burned N = 60
Cenococcum geophilum	22.75 B	3.17 A	2.20 A
Piloderma byssinum	15.53 B	0.13 A	1.00 A
Cortinarius spp.	0.92 A	0.00 A	0.00 A
Suilloids	17.9 A	4.75 A	7.13 A
Suilloid unknown spp.	6.93 A	0.00 A	1.32 A
Suillus spp.	1.17 A	0.00 A	3.28 A
Rhizopogon spp.	9.80 A	4.75 A	2.53 A
Amphinema byssoides	1.78 A	4.33 AB	11.87 B
Wilcoxina spp.	1.45 B	17.42 A	20.27 A
Pseudotomentella nigra	0.92 A	7.42 A	38.87 B
Thelephoroid spp.	0.00 A	0.00 A	4.76A

Tukey's HSD analysis taxa with significantly different abundances between burned and unburned areas without regard to planting/natural regeneration are *Wilcoxina* spp., *C. geophilium*, and *P. byssinum.* In contrast, the abundance of *P. nigra* in the planted burned group is significantly higher than for either the natural unburned and planted burned groups. The abundance of *A. byssoides* under the different conditions shows the dual impact of planting and burning: while there is a significant difference between natural unburned and planted burned seedlings groups there is no difference for natural burned seedlings with the other conditions. For some taxa, such as those in the suilloid groups, *Cortinarius* and Thelophoroids, no significant differences were found among any group, possibly due to their low occurrence among seedling groups and high variation.

Analysis of Fungal Communities:

Comparisons were made among seedling groups to determine if differences in individual taxa would translate to differences in overall fungal communities. A

comparison of Shannon's and Simpson's diversity indices show that natural seedlings on the unburned area have the highest diversity, next are natural seedlings on the burn and seedlings planted on the burn have the lowest diversity (Table 2.7). Analysis of Variance tests found that natural seedlings in the unburned area have a significantly higher diversity than both natural and planted seedlings in the burn using Shannon's diversity index. An ANOVA using Simpson's diversity index found that natural seedlings in the unburned area have a significantly higher diversity than seedlings planted in the burn, but neither is different from seedlings naturally regenerating in the burn.

Table 2.7: Average Shannon's and Simpson's diversity of whitebark pine seedlings by condition and significance groupings.

Group	Shannon	Simpson
Natural Unburned	0.56 A	0.37 A
Natural Burned	0.32 B	0.21 AB
Planted Burned	0.21 B	0.16 B

Kruskal's Non-metric Multidimensional Scaling (NMDS) was performed to illustrate any patterns in fungal community composition by seedling grouping. The correlation coefficient for the mapped distances in the ordination plot and the calculated dissimilarity values is 0.84. The first two axes of the mapping displays a grouping of planted and naturally regenerating whitebark pine seedlings in the burned area separated from naturally regenerating seedlings in the unburned forest (see Figure 2.8a). The treatments of burned or unburned explains 60% of the deviance in the mapping. The unexplained deviance can be seen in axis-2 of figure 2.8a for seedlings growing in the burned areas and in the fourth dimension plotted on axis-2 for the seedlings growing in unburned areas in figure 2.8b.

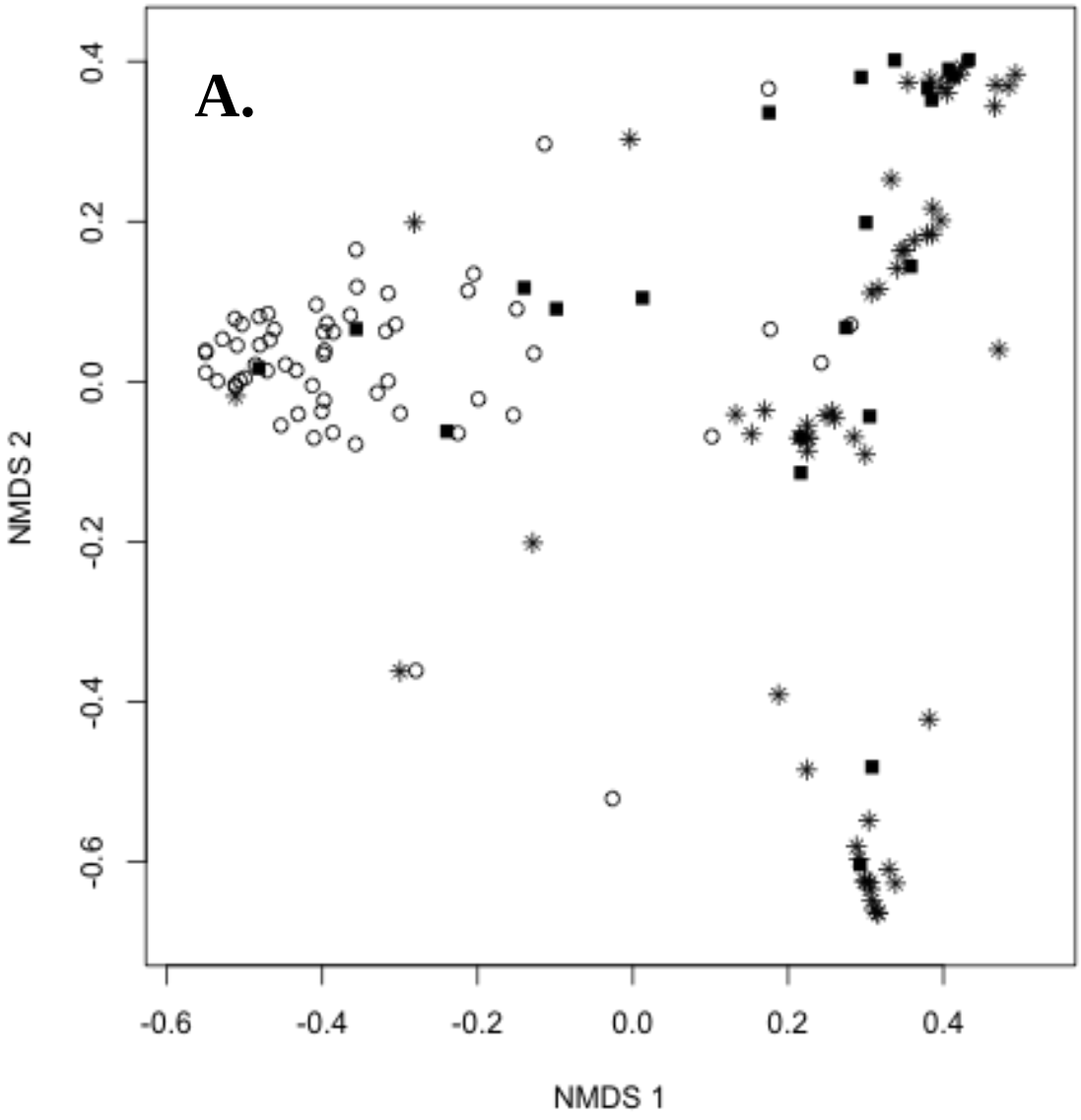


Figure 2.8A: Heading on page 48.

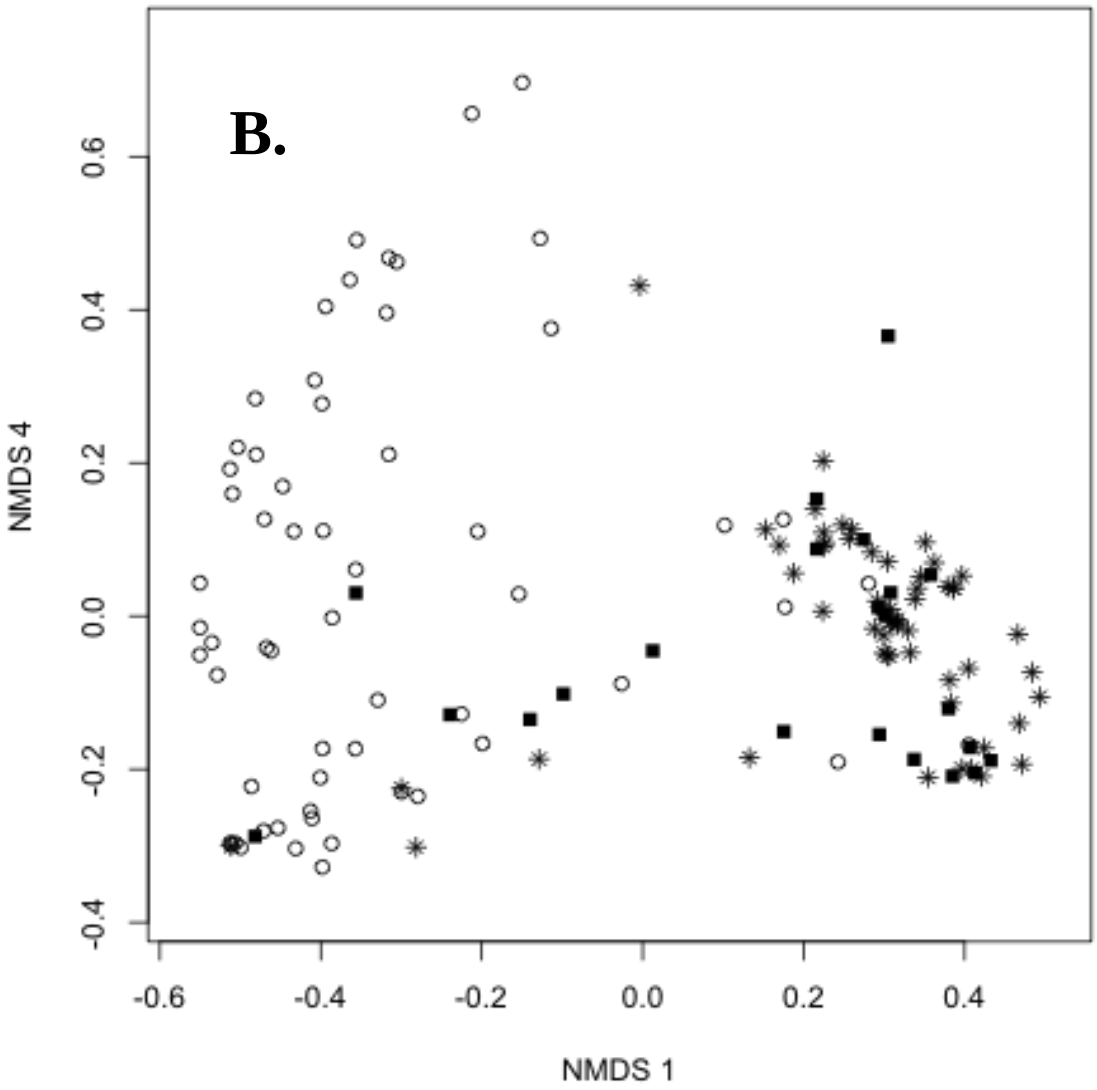


Figure 2.8: Non-metric Multidimensional Scaling of Bray-Curtis dissimilarity matrix of ectomycorrhizal communities separated by group status (Natural Unburned - circles, Natural Burned - asterisks, Planted Burned - squares). A: NMDS mapping of dimension one on the x-axis and dimension two on the y-axis. B: NMDS mapping of dimension one on the x-axis and dimension four on the y-axis.

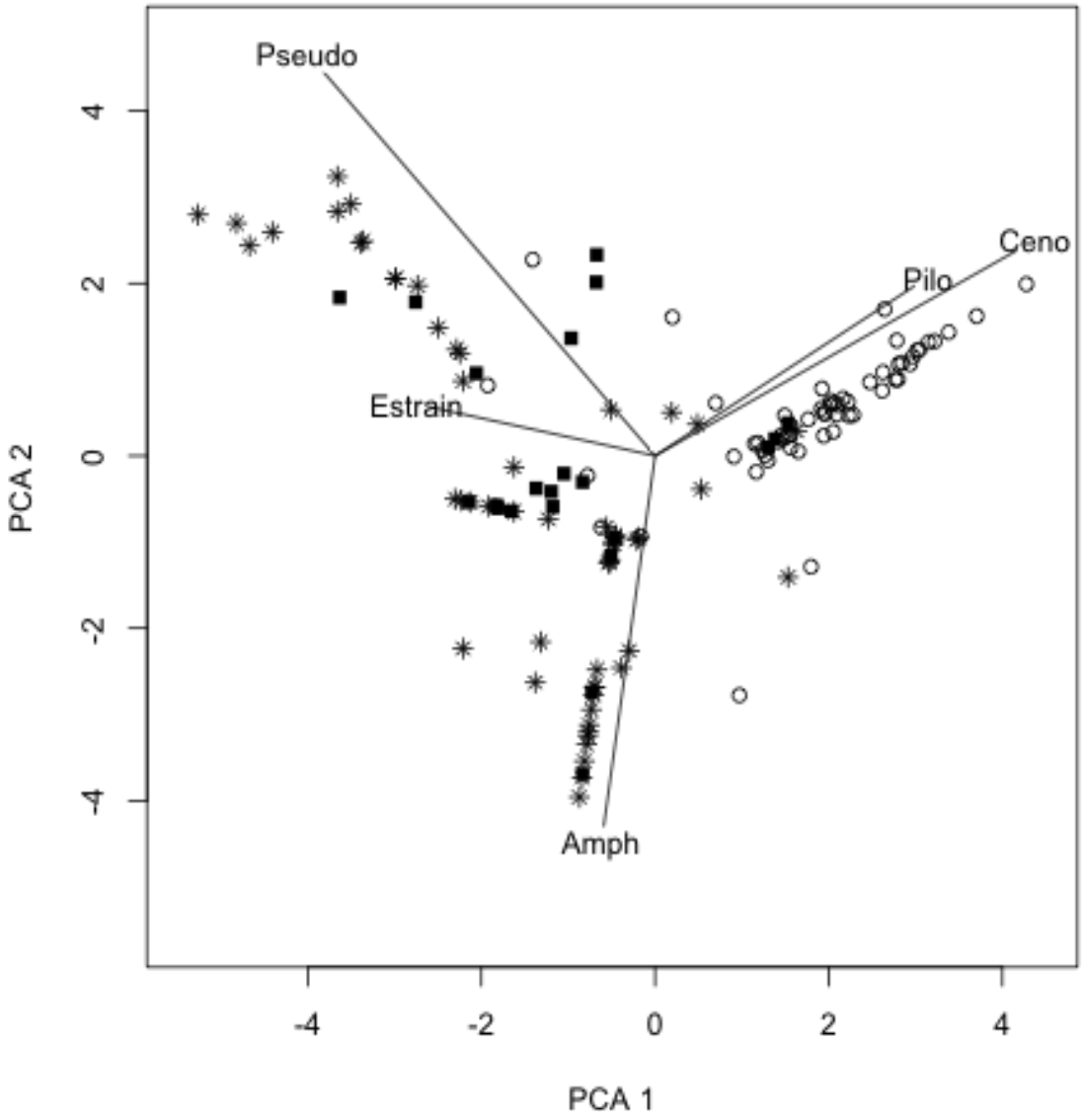


Figure 2.9: Principal component analysis plot of log transformed ectomycorrhizal species abundances. Only loading vectors for species with values over 0.1 are shown. Circles are natural unburned seedlings, black squares are natural burned seedlings, and asterisks are planted burned seedlings. Pseudo= *Pseudotomentella nigra*, Estrain= E-strain species, Pilo= *Piloderma byssinum*, Ceno= *Cenococcum geophilum*, Amph= *Amphinema byssoides*.

The PCA plot and loading scores vectors show that the seedlings growing in burned areas are grouped together regardless of planting status. E-strain species, *A. byssiodes*, and *P. nigra*, are mainly associated with the seedlings growing in the burned

area. Seedlings in the unburned whitebark pine forest have higher abundances of *C. geophilum* and *P. byssinum*. The PCA plot was included for visualization even though the NMDS plot is a more accurate reflection of the calculated distances because it allows for the plotting of loading scores which allows for interpretation of species that are prominent under different environmental conditions.

A Multi-response Permutation Procedures test (MRPP) found that there is a statistically significant difference among the groups (p-value < 0.00005) when separated by seedling status (natural burned, planted burned, natural unburned). A pairwise comparison of all the seedling groups found that there were statistically significant differences between all groups (p-value < 0.05). MRPP was used as it functions similarly to ANOVA in finding differences among groups but also allows for the use of distance or dissimilarity measures. Also, it is a nonparametric procedure which allows some of the assumptions of parametric procedures such as ANOVA to be violated. Because it is a permutation based method the power of the analysis is not reduced as in other nonparametric procedures.

Discussion

The long-term conservation of whitebark pine communities may be tied to the regeneration and out-planting of whitebark pine seedlings. The continued loss of susceptible whitebark pine trees to white pine blister rust and mountain pine beetles has necessitated the active restoration of these forests. Current restoration activities are based on the reintroduction of fire which initiates the natural regeneration of whitebark pine

seedlings and allows space for out-planting of resistant seedlings. This study provides information on the impact of fire on the ectomycorrhizal communities of whitebark pine seedlings under burned, unburned, planted, and natural conditions which could be important to these efforts.

Whitebark Pine Seedling Regeneration and Survival After Fire:

The regeneration and survival of whitebark pine seedlings inside burned areas has been examined in both the GYE and western Montana (Tomback et al. 2001, Perkins 2004, Keane and Parsons in press). In Yellowstone National Park it was found that whitebark pine seedlings have a two-year delayed seed germination, and germination rates and survival numbers were not different for burned and unburned areas in the 1988 fire but moisture had a positive affect (Tomback et al. 2001). In contrast, Perkins (2004) found that regeneration in a burned area of the Bitterroot Mountains of western Montana increased survival and growth compared to unburned controls. Also in the Bitterroot mountains, regeneration has been slow to occur due to low numbers of cone-producing mature whitebark pine trees in near proximity, despite the increased numbers of favorable caching sites (Keane and Parsons in press).

Vaccinium and Pine Mycorrhizae:

Perkins (2004) suggested that whitebark pine might share ericoid mycorrhizae with pre-established *Vaccinium* species which could serve to promote the regeneration of the pine. In her study, whitebark pine seedlings planted with *Vaccinium scoparium* had increased survival and growth rates. However, it was not possible for her to determine if

whitebark pine seedlings benefited directly from shared mycorrhizal connections. Perkins also suggested that *Vaccinium scoparium* might facilitate survival and growth of whitebark pine seedlings through increased phosphorus availability.

In the present study, molecular analysis found only one root tip to host a fungus closely related to the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (237/264 base pair similarity of ITS region) which was labeled as an unknown fungus because of its low basepair overlap. According to this study, the presence of ericoid fungi on whitebark pine seedlings is not a common event, even in the unburned whitebark pine forest which supports an extensive *Vaccinium* understory.

The ericoid fungus *Hymenoscyphus ericae* has been found on other conifers such as *Picea abies*. However, in re-synthesis experiments these isolates did not form mycorrhizae with the local *Vaccinium* species. Conversely, *H. ericae* strains from *Vaccinium* did not form mycorrhizae with *Picea abies* (Vralstad et al. 2002). This suggests there may be hidden diversity within this group of fungi. Villarreal-Ruiz et al. (2004) provide the first actual report of an ericoid fungal isolate of *H. ericae* to form mycorrhizae with both *Vaccinium* and *Pinus.* The pine isolate was synthesized *in vitro* with sterilized seedlings of *Vaccinium myrtillus* and *Pinus sylvestris*, so the association was in an artificial system. A few ectomycorrhizal fungi associated with conifers have been found in small quantities on the local ericaceous plants *Gaultheria* and *Rhododendron* (Smith et al. 1995), but this study also did not find colonization of conifers by ericoid fungi.

While there is some evidence that ericaceous and coniferous plants might share mycorrhizal fungi on a small scale, it is not clear that this is a beneficial arrangement. Haselwandter (1997) reviewed this topic and reports that spruce planted in ericaceous heathlands were infected with pathogenic not ericaceous fungi (Read et al. 2000). Moser (1963) showed that the survival rate of *Pinus cembra* (a stone pine related to *Pinus albicaulis*) planted in dwarf heath shrub (an ericaceous environment) was increased by inoculation with appropriate *ecto*mycorrhizal fungi. From the present study, it does not appear that sharing of fungi by *Vaccinium* and *Pinus* is common or functionally significant.

Ectomycorrhizal Colonization of Whitebark Pine Seedlings:

Root systems of all seedling groups were well colonized by ectomycorrhizal fungi (90-99%) four years after the fire. Neither burning nor planting had an effect on overall colonization of root tips. Root systems of nursery-grown whitebark pine seedlings planted on the burn were larger than those naturally regenerating in the burned or unburned areas. Seedlings were of comparable ages across treatments but nursery growing practices resulted in higher root and shoot biomass for planted seedlings. Root systems of nursery seedlings were still in a containerized shape four years after the fire, while natural regenerating seedlings roots spread in a more erratic and less dense pattern. The study was not able to examine how quickly seedlings were colonized after the burn and if there was a significant lag time. A study in a *Pinus muricata* forest in California (Baar et al. 1999) found that ectomycorrhizae formed less then six months after a stand-replacing wild fire.

While all seedling groups were well-colonized by ectomycorrhizal fungi, it is important to recognize that the planting strategy for this site was highly conducive to mycorrhization of whitebark pine seedlings **(**Wiensczyk et al. 2002). Aspects of the planting strategy likely to enhance post-fire ectomycorrhizal colonization were: 1) the existence of mature whitebark pine trees in an old growth forest in close proximity (potential inoculum source), 2) seedlings were planted in a burned area that was previously whitebark pine (potential spore bank), 3) plantings took place soon after the fire (less decline of inoculum), 4) ectomycorrhizal fruiting bodies were present in nearby mature forest (definite inoculum source), and 5) animal dispersal agents were present for spore dispersal. These factors help ensure availability of appropriate mycorrhizal fungi for whitebark pine.

Approximately 22 taxa of ectomycorrhizal fungi were identified on over 22,000 root tips of the whitebark pine seedlings examined in burned and unburned areas of this subalpine Gallatin National Forest study site (Table 2.3). The only other study that examined ectomycorrhizal fungi associated with whitebark pine throughout the Greater Yellowstone Ecosystem (across five mountain ranges) confirmed 32 species of ectomycorrhizal fungi with this pine by sporocarp identification or ITS sequence matching; of these, 19 were confirmed on seedling roots (Mohatt et al. 2008). None of these sites contained burns or involved planted seedlings. The present study confirms several of these ectomycorrhizal fungi on seedling roots and adds several new species to this list, including: *Amphinema byssoides*, *Piloderma byssinum*, *Pseudotomentella nigra*, *Thelephora terrestris*, *Wilcoxina rhemii*, and *Wilcoxina mikolae*. All except *Piloderma*

were associated primarily with the burn and the last three are commonly associated with nursery grown seedlings.

The present study reports a mean of 2.17 species per seedling in the unburned forest (Table 2.4), which is comparable to the 2.13 species per individual whitebark pine seedling found by Mohatt et al. (2008) for five mountain ranges in the GYE. As also reported by Mohatt et al. (2008), *Cenococcum geophilum* and suilloid fungi were two of the most common taxonomic groups of ectomycorrhizal fungi on whitebark pine seedlings regenerating naturally within mature whitebark pine forests. Importance values for *C. geophilum* and suilloids in the current study are 0.78 and 0.50 respectively (Figure 2.7), and about 0.80 and 0.40 for Mohatt et al. (2008). *Cenococcum geophilum* is a generalist fungus found with many tree species. *Cenococcum geophilum* is mainly found in closed canopy forests but is often persistent, if reduced after disturbances (Visser 1995, Izzo et al. 2006b). The suilloid taxa (*Rhizopogon* and *Suillus*) discovered are in taxonomic groups specific for pines, and some are possibly restricted to five-needle pines (Massicotte et al. 1994).

A contrast between this study and Mohatt's (2008) for seedlings in unburned forests is the lower level of *Cortinarius* species reported in the present study. The diversity of *Cortinarius* species in Mohatt et al. (2008) was primarily reported as sporocarps and it is likely that these are associated primarily with mature trees. While *Piloderma byssinum* was common in the present study, it was not reported in Mohatt et al. (2008). *Piloderma* has subsequently been reported in mature whitebark pine forests in Yellowstone, Glacier, and Waterton Lakes National Parks (Trusty and Cripps 2007).

Piloderma byssinum is generally thought to occur within the canopy of mature forests (Smith et al. 2000, Bergemann and Miller 2002, Smith et al. 2005) as in the present study. *Piloderma* is also a generalist ectomycorrhizal fungi known to associate with a variety of conifers, including Engelmann spruce and subalpine fir (Dunham et al. 2007).

Comparison of Ectomycorrhizal Communities:

Ordination analysis has been found to be a useful tool for examining ectomycorrhizal communities (Figures 2.8 and 2.9). It has been used to compare fungal communities at timberline/alpine ecotones (Kernaghan and Harper 2001) and to examine the effects of thinning on ectomycorrhizal communities (Shaw et al. 2003). By using Bray-Curtis dissimilarity distances with Multiple Response Permutations Procedure, we were able to examine the effects of planting and fire on communities of ectomycorrhizal fungi on whitebark pine seedlings. These analyses determined that the communities of ectomycorrhizal fungi differed significantly among all three groups of seedlings (naturally regenerating on unburned soil; naturally regenerating on burned soil; planted on burned soil). Differences are a result of changes in multiple factors including: taxa frequency, taxa abundance, species richness per seedling, and overall diversity. However, some taxa were found to be equally common on all seedling groups four years after fire.

When the Non-metric Multidimensional Scaling mapping was fitted for the environmental variable of whether the seedling was growing in a burned or unburned forest, it explained 60% of the deviance. The rest of the deviance is unexplained by environmental variables. More in-depth work is needed to determine which factors might explain this residual difference in the fungal communities. Many biotic and abiotic

factors differ between burned and unburned forests (Neary et al. 1999). Plant, animal, and soil micro-organism species can change dramatically after fires. Abiotic characteristics such as carbon-nitrogen ratios, soil mineral content, and soil structure are altered. Any of these factors could potentially influence the ectomycorrhizal fungal communities of whitebark pine. In order to determine more specifically what is driving these changes, a wide range of burned and unburned whitebark pine forest would need to be examined. Variables are confounded with only one research site, such as in this study. Soil temperature was significantly warmer for the burned soil but this is just one of many factors, so it is not possible to say with reliability that it is the prime reason for a change in ectomycorrhizal fungal communities.

Impact of Fire on the Ectomycorrhizal Community:

Fire has long been recognized for its critical role in maintenance and regeneration of whitebark pine forests (Tomback et al. 2001). Fire reduces competition from shade tolerant tree species, opens areas for nutcracker seed caching and seedling growth, and has been shown to increase whitebark pine seedling survival in comparison to seeds planted in unburned areas (Perkins 2004).

Fire can potentially impact the ectomycorrhizal community through a number of different mechanisms. “Fire, depending on the severity, can alter hydraulic properties of soil, change soil physical structure, sterilization of soil micro-organisms, loss of host species, loss of organic matter, altered nitrogen availability, and many other mechanisms” (Neary et al. 1999).

While numerous studies have examined the impact of fire on the ectomycorrhizal communities of other tree species, none have extensively examined the ectomycorrhizal fungi associated with whitebark pine seedlings regenerating on burns. Perkins (2004) studied regeneration of whitebark pine seedlings in burned areas and attempted to examine the potential connection of mycorrhizae between *Vaccinum scoparium* and whitebark pine seedlings on burned areas in the Bitterroot Range of western Montana. She did report *Cenococcum geophilum* and dark septate hyphae associated with roots of whitebark pine seedlings, but did not study the mycorrhizae in more detail.

The Fridley fire significantly reduced the diversity and species richness of the ectomycorrhizal communities on whitebark pine seedlings, and this reduction persisted for four years. In addition, there was an almost complete shift in species composition (primarily abundance) of the ectomycorrhizal community after the burn, regardless of planting status.

The impact of fire on ectomycorrhizal communities has been previously shown to be variable and influenced by the severity and time since the fire (Figure 3.6, Chapter 3), and effects are often site-specific (reviewed in Cairney and Bastias 2007). Dahlberg et al. (2001) found that ectomycorrhizal abundance and diversity decreased with increasing depth of burn (a measure of severity) in a boreal scots pine forest in Sweden. When the time since a burn was taken into account for jack pine stands six to 122 years post fire, ectomycorrhizal diversity increased significantly from six to 41-year-old stands (Visser 1995). Prescribed fire can also reduce the species richness of ectomycorrhizal fungi as

shown for a mixed conifer forest (Smith et al. 2005) and a ponderosa pine stand in the Blue Mountains of Oregon (Smith et al. 2004).

On the Fridley burn, there were significant reductions in the abundance of the dominant unburned ectomycorrhizal fungi *Cenococcum geophilum* and *Piloderma byssinum*. This is consistent with previous research that found *Cenococcum geophilum* abundance significantly lower on planted *Picea engelmannii* and *Picea glauca* seedlings in cut-burned areas in comparison with naturally regenerating seedlings in mature forests (Mah et al. 2001). The reduction appeared to be correlated solely with burning, as their abundance was not reduced on naturally regenerating, or planted seedlings in clear-cuts alone. In three Australian sclerophyll forests subjected to prescribed burning, *C. geophilum* was the main pre-fire species but could not be found post-fire (Chen and Cairney 2002).

Cenococcum geophilum has been shown to have a reduced ability to infect seedlings after severe heating. *Cenococcum geophilum* colonization of *Pinus jeffreyi* seedlings was significantly reduced in a bioassay of old growth mixed-stand soil after it was heated to the maximum tested temperature of 75°C before planting (Izzo et al. 2006a). However, in this study the high temperature was reached by heating not burning; other factors could possibly influence colonization by *C. geophilum* in nature after fire.

Lower levels of *C. geophilum* can also be tied to a lack of mature trees in close proximity. For *C. geophilum* on *Picea engelmannii* and *Abies lasiocarpa* seedlings (<1yr) and juveniles (2-10 yrs) at tree line, colonization decreased four-fold in seedlings farther than seven meters from mature trees (Hasselquist et al. 2005). This indicates mature trees

are likely a source of *C. geophilum* for seedlings, and since this fungus does not produce spores or fruiting bodies, direct contact is assumed to be the main method of transmittance. The lack of living mature trees in burned areas of our study site or in close proximity likely restricted *Cenococcum geophilum* from rapidly colonizing whitebark pine seedlings in the burn. However, while *Cenococcum geophilum* is often reduced after fires, in another study it was maintained at moderate frequencies after burning in a ponderosa pine forest (Stendell et al. 1999).

The abundance of *Piloderma byssinum*, another dominant fungus in the mature unburned forest, was also significantly reduced inside the burned areas. Research by Visser (1995) found that *Piloderma byssinum* did not occur in boreal jack pine stands until 41 years after fire and its abundance increased after that point until 65 years after the burn. Similarly, *Piloderma byssinum* abundance was reduced over ten times on naturally regenerating seedlings in clear-cut areas versus mature forests and it was not found at all on seedlings planted inside areas that were clear-cut or clear-cut and burned (Mah et al. 2001). In comparisons of multiple age class forests, *Piloderma byssinum* has been determined to be a late stage fungus of mature forests (Visser 1995, Torres and Honrubia 1997). In a microhabitat study of ectomycorrhizae on seedlings in a mature *Pinus sylvestris* boreal forest in Finland, *Cenococcum geophilum* and a species of *Piloderma* were both found to be common in the forest soil (Iwański and Rudawaska 2007). *Piloderma* species are known to associate with mature conifer forests that have a high amount of advanced decayed wood and organic matter (Danielson 1984, Goodman and Trofymow 1998a, Goodman and Trofymow 1998b, Smith et al. 2000, Bergemann and

Miller 2002, Smith et al. 2005). *Piloderma* may exist in areas of high organic matter, such as mature forests, because of a possible ability to degrade wood and it is reported to have laccase-like genes (Chen et al. 2003). The loss of *Piloderma* species appears to follow the loss of mature trees and decaying organic matter after severe burns and other disturbances (Visser 1995, Smith et al. 2005). Our study site is characterized by a complete loss of living mature trees and nearly complete loss of organic matter in the burned areas, which is in agreement with this hypothesis.

In our study, the most frequent and abundant taxa on naturally regenerating whitebark pine seedlings in the burn were *Wilcoxina* species (E-strain), *Pseudotomentella nigra*, suilloid fungi, and to a lesser extent *Cenococcum geophilum.* These results concur with previous research that reports *Wilcoxina rehmii* to be the most common species after fire, for example on ponderosa pine seedlings less than a year old (Fujimura et al 2005). In another study, *Amphinema*, *Wilcoxina*, and *Pseudotomentella* were both frequent and abundant after fires while *Cenococcum* was found to be moderately frequent but low in abundance (Purdy et al. 2002), matching the results of this whitebark study. In a greenhouse bioassay using *Pinus halepensis* seeds, suilloid species, (*Rhizopogon* and *Suillus*) along with *Wilcoxin* (E-strain) were found to be common in burned soil (Torres and Honrubia 1997). The surprising congruence of ectomycorrhizal taxa in recently burned habitats across different host tree species suggests a potential ecological or temporal niche inhabited by these species.

Nursery seedlings planted inside the burned area were colonized most abundantly and frequently by *Pseudotomentella nigra, Wilcoxina* spp., *Amphinema byssoides*, and

suilloid ectomycorrhizae. Few studies have examined the ectomycorrhizal taxa associated with out-planted nursery seedlings, and only Perkins (2004) examined this superficially for whitebark pine seedlings. A study that compared the impacts of clear-cutting and broadcast burning on the ectomycorrhizal fungi of planted *Picea engelmannii* and *Picea glauca* seedlings found *Amphinema* and *Wilcoxina* species to be common on planted seedlings in both cut and burned sites (Mah et al. 2001). Examination of the ectomycorrhizal fungi associated with nursery stock of *Picea glauca* grown in peat/vermiculite containers, a nursery condition similar to that of whitebark pine, *Amphinema byssoides* and a species of *Thelephora* were found to be dominant (Kernaghan et al. 2003), suggesting that the source for these ectomycorrhizal fungi may be the nursery.

The abundance of *Wilcoxina* spp. (E-strain) was significantly increased in burned areas in the present study. This result is in accordance with Torres and Honrubia (1997) who found that E-strain (*Wilcoxina spp.*) was more frequent after a burn in a *Pinus halepensis* forest. Field studies (Taylor and Bruns 1999) and bioassays (Baar et al. 1999) of *Pinus muricata* also report *Wilcoxina* as a dominant taxon following a stand-replacing wildfire. Examination of ectomycorrhizae communities over time after fire in a jack pine stand, found E-strain (*Wilcoxina spp.)* 6 years after fire but not in the 41 year old stands (Visser 1995). The increase in *Wilcoxina* after a fire is thought to result from production of thick walled chlamydospores by species that can survive fire to recolonize seedlings (Visser 1995). However, in a test on the effect of heat treatments on ectomycorrhizal propagules using a bioassay of forest soil planted with *Pinus jeffreyi* seeds, an unnamed

species of *Wilcoxina* was significantly reduced in frequency at 75°C (Izzo et al. 2006a). Alternatively, *Wilcoxina* species may increase in burned areas as an aggressive colonizer that thrives in environments such as burns, greenhouses, or after disturbances with few competitors (Scales and Peterson 1991, Torres and Honrubia 1997). It is not known if these species have the ability to survive the high heat of forest fires or are rapid colonizers on burns. E-Strain is common in nursery environments which suggests that a lack of competitors or particular environmental conditions favor its proliferation (Yu et al. 2001).

Several taxa of ectomycorrhizal fungi recorded on the whitebark pine seedlings planted on the burn are also known to originate in nurseries (Yu et al. 2001) including *Wilcoxina* (E-strain), *Thelephora terrestris*, and *Amphinema*. The first two have been molecularly identified on whitebark pine seedlings from the Coeur D'Alene nursery, although colonization rates were low (Cripps and Trusty 2007). Fruiting bodies of *Wilcoxina mikolae* were noted in a separate lot of seedlings from this nursery after transfer to the Montana State University Plant Growth Center. However, *Amphinema byssoides* and *Wilcoxina* species occurred on both planted and natural seedlings in the burn which suggests that these species also occur naturally in the area. The origin (native or nursery) of *Amphinema byssoides* and *Wilcoxina* species on seedlings planted on the burn could not be confirmed for this study. The resolution of the ITS region did not allow detection of differences in strains of *Wilcoxina* spp. for natural and planted seedlings.

Influence of Seedling Origin on ECM Community:

In addition to the impacts of fire, the ectomycorrhizal communities on natural and planted seedlings within the burn were compared. A primary difference was the significantly higher abundance of *Pseudotomentella nigra* on planted whitebark pine seedlings compared to naturally regenerating seedlings on the burn (and in unburned areas). Fruitings of *P. nigra* have been reported on charred conifer wood in Idaho (Ginns and Lefebvre 1993) showing a possible association with burns. An unknown species of *Pseudotomentella* was similarly reported after burning when the effects of prescribed burning on the ectomycorrhizal fungi of ponderosa pine stands in the Blue Mountains of Oregon were examined (Smith et al. 2004).

Pseudotomentella nigra has also been found on whitebark pine seedlings in Glacier National Park in open areas separated from the forest canopy (Cripps and Trusty, unpublished) and an unknown *Pseudotomentella* species occurred in narrow strips of whitebark pine between avalanche paths in the GYE (Mohatt 2006). An investigation in western Washington showed that when *Pseudotsuga meneszii* seedlings were planted at various distances from mature trees, those planted farther away (16-30 meters) had a higher relative abundance of *Pseudotomentella nigra* ectomycorrhizae than those planted within two to six meters (Cline et al. 2005, Cline et al. 2007). These findings suggest that *P. nigra* more commonly infects seedlings away from mature trees than those within enclosed canopies. This pattern is characteristic of early colonizing fungi; however, in contrast, this species appears to have need of a wood substrate for fruiting. Results from this study follow these same trends, however it is not clear why *P. nigra* was significantly

more abundant on planted seedlings than on natural seedlings in the burn. We speculate that this could be a result of the larger available root systems for nursery grown seedlings which might promote rapid colonization by *P. nigra*. The functional significance of higher levels of *P. nigra* on planted seedlings is not clear at this point.

Dual Impacts of Fire and Regeneration:

The largest significant differences in the three ectomycorrhizal communities occur between those on natural seedlings in the unburned forest and those on seedlings planted in the burn. Intermediate to these is the ECM community on natural seedlings in the burn which shares characteristics with each of the other groups. The importance values for four out of the six major ectomycorrhizal taxa in the study reveal an intermediate ECM status for seedlings regenerating naturally in the burn. For example, the abundance of *Amphinema byssoides* on natural seedlings in the burned area is not significantly different from either of the other two treatments. However, seedlings planted in the burn have a significantly higher abundance of *Amphinema* mycorrhizae than natural seedlings in the unburned forest. This suggests that the presence of *Amphinema byssoides* is slightly influenced by both planting and fire, and that the combined factors result in a significant difference. Mah et al. (2001) found a higher abundance of *A. byssoides* on planted *Picea engelmannii* and *Picea glauca* seedlings in both cut-burned and clear-cut forests in comparison to naturally regenerating seedlings in mature forests. In addition, they found the abundance of *A. byssoides* to be higher on naturally regenerating seedlings in the cut-burned site compared to those in the mature forest, but differences were not significant. These results suggest that *Amphinema*

byssoides may be more common on planted than natural seedlings and that levels increase after disturbance (including burns) or in areas away from mature trees.

Disturbance Resistant Taxa:

Suilloids (*Rhizopogon* and *Suillus* species) are known to be important ECM associates of whitebark pine and especially with seedlings (Mohatt et al. 2008). In this study, there appears to be a more uniform occurrence of suilloid ectomycorrhizae across all seedling groups four years after the fire, in contrast to the extreme changes in relative frequency and/or abundance of the main generalist ectomycorrhizal taxa discussed previously. Taken as a whole suilloids have a higher importance value in the unburned forest (0.50) in contrast to the seedlings in the burn (0.25 for naturals and 0.18 for planted). Statistical analysis of ectomycorrhizal abundance for suilloids results in a p-value of 0.06 for the three groups with a trend towards a higher number of suilloid mycorrhizae on seedlings in the unburned forest.

Table 2.8. Frequency, abundance and importance values of suilloid mycorrhizae on natural pine seedlings in the unburned forest and for natural and planted seedlings on the burn.

	Frequency	Abundance tips/seedling	Importance Value
Unburned & natural	0.21	17.9	0.50
Burned & natural	0.13	4.75	0.25
Burned & planted	0.10	7.13	0.18

Rhizopogon species have been reported in both pre- and post-fire ECM communities (Kjøller and Bruns 2003, Izzo et al. 2006b, Smith et al. 2005). In a field study in Oregon, *Rhizopogon salebrosus* A.H. Sm. was frequent both before and one to two years after prescribed burning in a ponderosa pine stand (Smith et al. 2004).

Rhizopogon species also occurred on pre-wildfire bishop pine (*Pinus muricata*) seedlings and on post-fire seedlings within five months of a stand-replacing fire in California (Horton et al. 1998). A greenhouse bioassay of burned and unburned soils from a *Pinus halepensis* forests found *Rhizopogon* species to be common in all soils (Torrres and Honrubia 1997).

Rhizopogon and *Suillus* species have been found to rapidly colonize post-fire and seedlings establishing away from mature forests. This rapid colonization is apparently brought about through two main mechanisms, resistant propagule communities (RPC) and mammal dispersal. *Rhizopogon* propagules were found to be well distributed throughout *Pinus muricata* and *Pinus ponderosa* forests according to greenhouse bioassays (Taylor and Bruns 1999, Kjøller and Bruns 2003). Resistant propagules (spores) were concluded to be the source of inoculum for naturally regenerating *Pinus muricata* seedlings that were colonized within five months of a stand-replacing wildfire (Horton et al. 1998). The frequency of *Rhizopogon olivaceotinctus* A.H. Sm. ectomycorrhizae increased in a greenhouse bioassay when soil collected from an old growth mixed-conifer forest was first heated to 75°C (Izzo et al. 2006a). It is not known if heat reduces competition or stimulates infectivity.

In addition, it is generally known that *Rhizopogon* fruiting bodies are eaten by small mammals that serve as vectors in spore dispersal (Johnson 1996, Luoma et al. 2004, Trappe and Claridge 2005). Ashkannejhad and Horton (2006) found that *Pinus contorta* seedlings establishing on sand dunes away from mature forests were colonized by a small subset of the taxa found in the mature forest and that seven out of ten RFLP

patterns were suilloid fungi. They also found that *Rhizopogon* and *Suillus* species dominated in a greenhouse bioassay of *Pinus contorta* seedlings inoculated with deer feces showing that large as well as small mammals disperse both *Rhizopogon* and *Suillus* spores. This whitebark pine research site supports many species of mammals including deer, elk, bears, and squirrels (per. obs.) known to disperse fungal spores. Also, a deer was observed eating *Rhizopogon* fruiting bodies and *Rhizopogon* spores have been confirmed in bear scat in whitebark pine forests in the GYE (Cripps, pers. comm.). These two mechanisms could explain the presence of suilloid ectomycorrhizae on seedlings in the burned area.

Suilloids and particularly *Rhizopogon* species are often important in regeneration of pines after disturbances (Kjøller and Bruns 2003). The presence of suilloids on planted and natural seedlings indicates that these potentially vital taxa were not lost after the burn and were able to form mycorrhizae on nursery-propagated seedlings. Suilloids are known to be restricted to particular host taxa, mostly pines, and some occur only with five-needle pines. At least five species of *Rhizopogon* and five species of *Suillus* are known to occur with whitebark pine in the GYE (Mohatt et al. 2008). *Rhizopogon evadens* and *R. milleri* are recorded with whitebark pine in the GYE (Mohatt et al. 2008) and both may be restricted to five-needle pines. In the present study, at least *Suillus variegatus* and *S. subalpinus* are similarly restricted to 5-needle pines, and the latter possibly to whitebark pine. The importance of individual suilloid taxa in the recovery process after fire is not known for whitebark pine.

Conditions at the site appeared conducive to seedling access to suilloid inoculum source in the adjacent forest and in the burned soil. Suilloids appeared to be reaching similar levels as the adjacent unburned forest four years after the fire. To speculate, the presence of suilloid mycorrhizae on all seedling groups could indicate a healthy whitebark pine system recovering after fire. However, the ectomycorrhizal community composition as a whole still differs significantly from the assumed pre-fire status four years after the fire.

Management and Conservation Implications:

In summary, root systems of whitebark pine seedlings were well colonized (over 90%) by ectomycorrhizal fungi in all treatments four years after the burn. However, fire reduced the species diversity and changed the composition of the ectomycorrhizal community on seedlings in the burn. There were minor differences in the ECM fungi on natural versus planted seedlings in the burn, and results show that both are colonized by the same post-fire fungal species. At this point, it is not known how a species shift and reduced diversity of ectomycorrhizal fungi affects seedling physiology and the long-term survival of whitebark seedlings after fire. These impacts could have implications in selection of restoration strategies for whitebark pine.

Given that fire is historically important in whitebark pine systems (Keane et al.1996a) it could be hypothesized that its associated mycorrhizal system is adapted to recover from this disturbance. In this study, fire reduced the assumed dominant fungi (*Cenococcum*, *Piloderma*) which were replaced by post-fire species (*Wilcoxina spp, Pseudotomentella nigra*, *Amphinema byssoides*). The former are species of mature forests

and the latter result from disturbance. There was also a trend towards reduction of suilloids after the fire, but this group did not show a dramatic shift. In her research in jack pine stands in Alaska, Visser (1995) found that diversity levels of ectomycorrhizae did not return to pre-fire levels until 41 years after the fire.

Planted and natural seedlings inside the burned area of the study site became naturally infected with suilloid species (at a frequency of 10% and 13% respectively), and this genus is known to be important in pine establishment. However, levels are still lower than assumed pre-fire frequency (25%) four years after the burn. This could be a typical time sequence in post-fire mycorrhization, at least where appropriate fungal inoculum is available (Visser 1995). A question that should be considered is whether whitebark pine forests can be sustained with only generalist mycorrhizal fungi in the absence of the more host-specific suiloid associates. Generalist fungi promote most conifers, while suilloids preferentially benefit pines over spruce and fir, likely giving pines a competitive edge and sustaining a pine climax condition. It is possible that the loss of fungi specialized for whitebark pine could tip the balance of tree species in high elevation western conifer forests. Unlike most other fungi specific for pine, suilloids can colonize both seedlings (at establishment) and mature trees (forest sustainability).

E-strain was present on all groups of seedlings, with significantly higher levels on seedlings in the burn; however it was not possible to delineate native versus any persistent nursery strains. There is presently no evidence to suggest that the nursery strain is problematic to colonization by native mycorrhizal fungi.

It should be re-emphasized that site conditions appeared optimal for the timely mycorrhizal colonization of suilloids. Previously, the burned area was an intact whitebark pine forest and it is adjacent to an extensive unburned mature whitebark pine forest. Both factors increase the probability that suilloid species can maintain a resistant propagule community in the soil and that propagules can be distributed by mammals transporting spores to new seedlings. In addition, seedlings were planted within one year of the fire and inoculum levels are known to decline over time (Wienszcyk et al. 2002). Planting seedlings in areas where whitebark pine has not previously existed, or in late seral stands where few whitebark pine exist, decreases the chances that suilloid fungi are available as inoculum for seedlings (Wiensczyk et al. 2002). For whitebark pine, this would include plantings in ghost forests where live whitebark pine have not existed for years.

Other concerns for management could include the temperature and timing of prescribed burns. Mathiasen and Albion (2001) found that both prescribed and severe wildfire in a northern Arizona ponderosa pine forests reduced the number of ectomycorrhizal species and they concluded that wildfire should not be used exclusively to improve forest health. In the present study, diversity was reduced on whitebark pine seedlings from 0.56 to 0.32 for natural and 0.21 for planted seedlings after the Fridley fire which initiated August 19th 2001. For ponderosa pines in the Blue Mountains of Oregon, Smith et al. (2004) found that there was a significant reduction in ectomycorrhizal species richness in fall prescribed burns in comparison to spring burns or unburned stands. A future concern is a possible general increase in burn severity due to previous fire suppression and/or climate change which typically correlates with a reduction in

mycorrhizal fungi. The Fridley fire was severe and scorched the soil, and while mycorrhization levels were high, diversity remained substantially reduced four years after the burn. We would expect light to moderate fires to have a smaller impact on ectomycorrhizal communities.

While mycorrhization is high on the site, Izlar (2007) found that survival levels of planted seedling were currently around 42%. This suggests that other factors (biotic and abiotic) are involved in seedling mortality or that the timing/type of mycorrhizal colonization can be problematic. Possible abiotic factors include changes in soil pH, nutrient availability, temperature and moisture, all of which are known to change mycorrhizal communities in the soil. During the study, temperatures were significantly higher (about 8^{o} C) on the burned area (Table 2.1), and this in itself is known to affect changes in the ectomycorrhizal community. Microhabitat and biotic factors were not addressed in the present study. Microhabitat is important in the survival of whitebark pine seedlings (Mellmann-Brown 2002, Izlar 2007) and Cripps et al. (2008) found that microhabitat might affect the diversity of mycorrhizal associations on naturally regenerating whitebark pine seedlings.

Study results showed a decrease in ectomycorrhizal diversity after burning, but as fire is a natural component of this ecosystem, it would be premature to conclude that this is a negative impact on the regenerative ability or overall forest health for whitebark pine. Fire is beneficial in opening up new areas for light-dependent whitebark pine seedlings and in reducing competitive tree species. It also opens up new areas where nutcrackers can plant seeds. At this point, it is not possible to assess the functional significance of this

shift in mycorrhizal species in terms of benefits/disadvantages to seedlings. The rapid decline of whitebark pine has initiated extraordinary measures such as cone collection for seed, selection of rust-resistant trees for planting, and the use of fire to open new areas (Schwandt 2006). Added to this should be a consideration of how mycorrhizal colonization will proceed, including information on the availability of suilloid fungi at the site and careful monitoring of the mycorrhizal status of planted seedlings. Conditions that contribute to optimizing mycorrhizal colonization of whitebark pine with appropriate fungi should be considered in all cases, since pines cannot survive in nature without this mutualism.

Future Possibilities:

This study found that wildfire and propagation method can impact the ectomycorrhizal communities of whitebark pine seedlings. It is for future research to discover how these changes in the mycorrhizal community might affect the physiology and survival of whitebark pine seedlings on burns. Monitoring the mycorrhizal status of planted seedlings can identify risky situations where appropriate mycorrhizal fungi are lacking. In this case, inoculation of rust-resistant seedlings in the nursery could be necessary. Inoculation is known to improve out-planted seedling health and survival under certain circumstances. Ponderosa pine inoculated with *Rhizopogon* and planted in harsh, dry sites in southwest Oregon demonstrated an increase in survival of at least 40% over non-inoculated seedlings (Steinfeld et al. 2003). Moser (1963) found that inoculation with ectomycorrhizal fungi benefited high elevation stone pines (*Pinus cembra*) in Europe where pines have been inoculated with suilloid fungi for over 50 years

with success (Austrian National Nursery, pers. comm.). In seral whitebark pine stands where succession has advanced to the point where few whitebark pine trees exist or where severe fire, blister rust or beetles have eliminated an entire stands, levels of appropriate inoculum for whitebark pine may be low and important species restricted to this pine may eventually be lost. Under these conditions, inoculation of nursery-propagated seedlings with native species can likely contribute to the long-term survival of out-planted whitebark pine seedlings.

Although there is a long battle ahead to ensure the long-term preservation of whitebark pine forests, this research adds information on how fire and regeneration strategy affect the mycorrhization process, which should be taken into account along with other factors in restoration of this species in peril that is currently being considered for endangered species status in the US and has already achieved a comparable status in provinces of Canada (C. Smith, Waterton Lakes National Park).

CHAPTER 3

A GREENHOUSE BIOASSAY TO DETECT ECTOMYCORRHIZAL FUNGI IN BURNED AND UNBURNED SOIL FROM WHITEBARK PINE SITES

Introduction

Whitebark pine (*Pinus albicaulis*) is the subject of concentrated restoration efforts in the Greater Yellowstone Ecosystem (GYE) and throughout its range in North American (Tomback et al. 2001, Schwandt 2006). The intense focus on whitebark pine restoration is necessitated by its dramatic reduction in area and its keystone species status in western montane forest systems (Tomback et al. 2001). Mortality of whitebark pine is up to 90% in some areas principally due to the invasive pathogen white pine blister rust (*Cronartium ribicola*) for which *P. albicaulis* has almost no natural resistance (Hoff et al. 2001). Mountain pine beetles are also a component in the reduction of populations (Kegley et al. 2001) and are becoming increasingly problematic in the GYE. In addition, decades of fire suppression have allowed whitebark pine's fire intolerant, shade tolerant competitors (*Picea engelmannii*, *Abies lasiocarpa*) to encroach and exclude whitebark pine regeneration, thereby slowly changing the species composition of these forests (Arno 2001).

Restoration efforts for maintaining whitebark pine forests are not only beneficial for retaining high-elevation biodiversity but also because this tree species plays key ecological roles in subalpine ecosystems (Tomback et al. 2001, Primack 2002). Whitebark pine is an early colonizer of harsh habitats such as post-fire openings and at

the alpine/subalpine border at treeline where it forms large, pure forests (Arno and Hoff 1990). After establishment, whitebark pine mediates the environment making it more hospitable for other organisms, initiating complex communities. Whitebark pine forests slow the melting of snow for a more constant release of moisture throughout summer and reduce spring flooding (Farnes 1990). In addition, whitebark pine seeds are a valuable source of food for many birds and animals, including the threatened grizzly bear (Tomback and Kendall 2001, Mattson et al. 2001).

Current restoration efforts are focused on creating openings for recruitment of whitebark pine seedlings followed by the out-planting of rust-resistant nursery propagated seedlings. Openings and planting areas are mainly established through fire, either by allowing natural fires to burn or through the use of prescribed burns. This appears to be beneficial as it eliminates competitor trees and creates space in which natural regeneration or planting of the pine can occur (Schwandt 2006).

In efforts to restore or recover whitebark pine forests, over 200,000 whitebark pine seedlings were planted between 1989 and 2005. Over 120 out-planting sites exist in the Rocky Mountains of Wyoming, Idaho, and Montana, many in national forests and parks such as Yellowstone and Glacier (McDonald and Hoff 2001, Izlar 2007). Large-scale and long-term research efforts such as the Restoring Whitebark Pine Ecosystems (RWPE) project are attempting to determine the most effective treatments to return these forests to their original structure (Keane and Arno 1996, Keane et al. 1996a, Keane and Arno 2001). The overall survival rate for the planted whitebark pine seedlings is low at 42% (Izlar 2007).

One aspect of restoration treatment for any pine species that is often overlooked is the availability of appropriate ectomycorrhizal fungi at the out-planting site. All pine species, including whitebark pine, are obligately mutualistic with ectomycorrhizal fungi and are not found in nature without them (Smith and Read 1997, Read 1998). It is hypothesized that the ectomycorrhizal fungi available in an area drive the plant species that can establish and influence plant/forest succession (Trappe and Luoma 1992). Potential restoration sites that have advanced in succession (to spruce-fir) so that few young whitebark pine trees exist may lack the early-stage fungi necessary for whitebark pine establishment since ectomycorrhizal fungi also follow a successional gradient (Trappe 1977, Molina and Trappe 1982, Allen 1992). Also, the high heat associated with fires and increased fire severity due to prior fire suppression could kill or reduce the inoculum potential of a site hindering regeneration of natural and planted seedlings (Cairney and Bastias 2007). The mycorrhizal inoculum potential of burned areas as well as clear-cut areas has been shown to decline within a few years (Harvey et al. 1979). The success of reforestation might be increased if managers are able to determine the ectomycorrhizal potential of a restoration site before out-planting thus saving time and money.

One solution is to perform a nursery bioassay to determine if the ectomycorrhizal fungi necessary for whitebark pine regeneration are present in the soil. For a bioassay, seedlings are planted in soil from a site and placed in greenhouse conditions. The seedlings are later examined for ectomycorrhizal colonization of the roots. A greenhouse bioassay can potentially pre-determine the inoculum potential of a restoration site (Torres

& Honrubia 1997, Baar et al. 1999, Bidartondo et al. 2001, Ashkannejhad and Horton 2006, Haskins & Gerhing 2005, Izzo et al. 2006b).

A bioassay can also provide insights into the local composition of the ectomycorrhizal community. However, we do not know if greenhouse bioassays accurately reflect the species diversity and abundance of ectomycorrhizal fungi in the field. Only limited research has compared results from bioassays with those for seedlings on field sites. These studies reveal varying degrees of consistency between field seedlings and bioassay seedlings in terms of species richness and abundance of individual species of ectomycorrhizal fungi. A bioassay of soil from a burned *Pinus muricata* forest in California yielded only four of the seven most abundant taxa found on field seedlings (Baar et al. 1999). Another bioassay of soil from *Pinus longaeva* forests in California found all four of the most common ectomycorrhizal fungi associated with field trees but these represented only 40% of all taxa that occur at the restoration site (Bidartondo et al. 2001). A study of primary succession in Oregon coastal sand dunes compared the ectomycorrhizal fungi associated with both field and bioassay seedlings from three eco-zones (mature, deflation, isolated) and found different levels of taxonomic overlap depending on the zone (Ashkannejhad and Horton 2006).

In future restoration efforts, it may be necessary to inoculate whitebark pine seedlings with appropriate native fungi before they are out-planted in areas that lack the necessary fungal inoculum and where it is not likely to be brought in by natural vectors such as small mammals or by wind from mature forests (Wiensczyk et al. 2002).

Therefore, it is critical to first determine if the ectomycorrhizal fungi important to the establishment of whitebark pine regeneration are present in the soil. Modern molecular techniques (ITS sequencing) serve the purpose of identifying these fungi on roots in the field and on bioassay seedlings in the greenhouse so comparisons can be made.

While several studies have used bioassay methods to determine the ectomycorrhizal fungi present in native soils for pines, no studies have addressed these questions for whitebark pine, a species for which substantive restoration efforts are now underway (Keane and Arno 1996, Keane et al. 1996a, Keane and Arno 2001). This study was undertaken to provide information on the potential for effective mycorrhization of whitebark pine seedlings by local native fungi in burned and unburned soil. It is a necessary first step for making treatment recommendations from a standpoint of scientific knowledge.

Goals of this research are to: 1) determine if a greenhouse bioassay is an effective tool for predetermining the presence of appropriate ectomycorrhizal fungi on a potential restoration site before large scale out-plantings of whitebark seedlings are initiated, 2) determine if results from the bioassay accurately reflect the diversity of the ECM community on seedlings planted in the field and 3) evaluate and suggest improvements for the bioassay technique itself.

Materials and Methods

Soil Collection and Preparation:

In June of 2006 soil was collected from burned and unburned areas of the Gallatin National Forest at the West Pine Creek Restoration Site described in Chapter Two (pp. 30-31). The burned area is a result of the 2001 Fridley Fire. Soil was collected from 1) the unburned whitebark pine forest, 2) the adjacent burned whitebark pine forest, 3) and a local burn site at a lower elevation, also previously covered with whitebark pine. One liter of soil was collected from each of ten sampling sites for the upper burned and unburned areas and from five sampling sites for the lower burned area. The soil was collected to a depth of ten centimeters and in the unburned area the surface duff was removed before collection of the soil. Soil was placed in plastic bags and transported back to the lab. The soil was then stored at 4^{o} C for later use.

The soil was sieved (5mm) to remove any large material but potential sources of inoculum, such as roots, were replaced into the soil. The soil sub-samples were combined for each treatment, resulting in two large soil mixtures, one from burned areas (upper and lower) and one from the unburned area. Half of each combined soil type was placed in a burlap sack and steam sterilized overnight for use in the controls. The sterilized and non-sterilized soil collected from each area was then diluted 50% with equal parts of autoclaved sand (mm) and peat as a mixture. Dilution was done to ensure proper drainage and to increase the probability of the formation of ectomycorrhizae by less common species of fungi (Taylor and Bruns 1999, Kjøller and Bruns 2003). Bioassay seedlings were planted in each of the three mixtures and in sterilized control soils.

Figure 3.1: Whitebark pine seedlings planted in field soils under greenhouse conditions.

Whitebark Pine Seedling Plantings:

Two year-old whitebark pine seedlings that had been grown under nursery conditions were obtained from the USDA Forest Service Nursery in Coeur D'Alene, Idaho. Seedlings were removed from small diameter conetainers and placed in larger diameter conetainers with the test soils. Seedlings were the same age as those out-planted at the site. Seedlings were examined for nursery mycorrhizae before being planted in the nursery field soil.

Ten seedlings were planted in each of the sterilized soils for the controls and ten in each of the unsterilized native soils for the treatments 1) upper unburned, 2) upper burned, 3) and lower burned soil (Table 3.1). Ten additional seedlings were planted in potting soil similar to typical nursery potting mix to determine if any nursery fungi were already present on roots. Seedlings were placed in greenhouse conditions and watered every other day to saturation. Seedling positions were rotated every week to avoid inconsistencies in watering or light. Seedlings were grown for one year: four months in a greenhouse, four months in a growth chamber at 10^{o}C for 16 hours, and for 8 hours at 4^{o}C for cold stratification and for another four months in the greenhouse.

Table 3.1: Soil treatments for bioassay seedlings planted in conetainers in the greenhouse.

Area	Fire Status	Sterilization	# Seedlings	Soil mix*
Upper	Burned	No	10	2:1:1
Upper	Burned	Yes	10	2:1:1
Upper	Unburned	No	10	2:1:1
Upper	Unburned	Yes	10	2:1:1
Lower	Burned	No	10	2:1:1
Nursery	Potting Soil	No	10	2:1:1

*soil:sand:peat

Identification of
Ectomycorrhizal Fungi on Greenhouse Seedlings:

After 12 months, seedlings were removed from the conetainers and examined for ectomycorrhizae. The entire root system was removed from the seedling and spray washed on a 500 µm sieve to rinse soil matter from the roots. The cleaned root system was then examined with a hand magnifier for ectomycorrhizal morphotypes (Bidartondo et al. 2001). Unique morphotypes were removed and saved in 2% CTAB at –20^{o}C for later molecular analysis.

After a representative sample of each unique ectomycorrhizal morphotype was removed and saved, roots were examined in sections taken from the top, middle, and bottom of each root system. Each sample of a 35-40 cm length of roots was spread in a single layer in a Petri plate filled with deionized water and examined with a dissecting microscope. Ectomycorrhizal root tips were recognized by the presence of a mantle, extramatricular hyphae or rhizomorphs, and the dichotomous branching typical of pines. For each sample, ectomycorrhizal tips were separated into morphotypes based on branching patterns, mantle color, and hyphal or rhizomorph characters (Agerer 1987-2006).

Samples of ectomycorrhizal tips suspected of being *Cenococcum geophilum* were examined more intensively with a light microscope for the distinctive star-shaped mantle pattern so that molecular identification would be unnecessary. Root tips of each morphotype were counted to determine abundances within each sample. Non-mycorrhizal and non-viable ectomycorrhizal root tips were also counted, and the latter were defined by a dark color and desiccated appearance due to low turgor. Each morphotype was catagorized according to its characteristics.

A representative sample of each ectomycorrhizal morphotypes was taken from each seedling and stored separately in a 1.5 mL centrifuge tube in 1mL of 2% CTAB and placed in a –20^{o}C freezer for subsequent molecular work. If more than 15 root tips of a morphotype were available, a voucher collection was placed in deionized water and saved at –20^{o}C. Identification through DNA fingerprinting of the ITS region was then carried out on the unknown ectomycorrhizal morphotypes following the procedures described in Chapter Two (pp. 31-36).

Analysis of the
Ectomycorrhizal Community on Field Seedlings:

Roots of whitebark pine seedlings were collected from the Fridley Burn restoration area during the summer of 2006. Samples were taken from sixty seedlings that were planted on the burn in 2002, from 60 seedlings naturally regenerating in the unburned area, and from 24 seedlings naturally regenerating in the burned area; all were examined for ectomycorrhizal fungi (see Chapter Two; pp. 25-36 for details). Root samples taken in the field were transported to the mycology laboratory at Montana State

University where they were processed for ectomycorrhizal identification in a manner similar to that used in the bioassay.

Statistical Analysis:

The survival of bioassay seedlings was compared for various treatments using a Fischer's Exact test. Treatments were first compared to each other and then all seedlings in burned soil (sterilized and unsterilized) were combined and compared to all seedlings in unburned soil (sterilized and unsterilized).

The relative abundance and frequency for the most encountered ectomycorrhizal fungal taxa were also determined and summed for importance values (Horton and Bruns 2001, Cripps 2004). Importance values were compared among all bioassay conditions.

The biodiversity of ectomycorrhizal fungi in each bioassay treatment was compared by calculating Shannon's diversity indice for each individual seedling followed by an Analysis of Variance and Tukey's HSD test. The biodiversity of bioassay seedlings growing in unsterilized burned soil and field seedlings planted in the burn was also compared. In addition, the biodiversity of bioassay seedlings growing in unburned unsterilized soil was compared to that for field seedlings naturally regenerating in the unburned area. The diversity comparisons between field and bioassay seedlings were done using individual Students t-tests.

All statistical analyses were carried out using the R statistical language (R Development Core Team 2009).

Results

A total of 8,163 root tips were examined from the 51 surviving seedlings of the original sixty. For soils that had been steam sterilized, 82% of the root tips were mycorrhizal and for the potting soil 2.6% of roots were mycorrhizal (Table 3.2). All ectomycorrhizal taxa found in sterilized soils and the potting soil were nursery type fungi, primarily E-strain (Table 3.3). For unsterilized native soils, 93% of root tips were mycorrhizal with both native and E-strain fungi present (Table 3.2). The frequency of native fungi on seedlings bioassayed in unsterilized ranged from 10 to 57%. The frequency of putative nursery fungi (E-Strain) was high (57-100%) in all soil types except potting soil (Table 3.3).

Table 3.2: Numbers and Percentages of ECM root-tips on whitebark pine seedlings in various bioassay soils. N = number of seedlings that survived to be assayed. Percentages are based on numbers of surviving seedlings. Native soil came from burned and unburned whitebark pine forests, near the Fridley Burn, Gallatin National Forest, MT.

Soil Type	N	Non ECM Root Tips	ECM Tips	Total Tips	% Mycorrhizal
Sterilized Native	17	531	2413	2944	81.96%
Upper Burned	10	176	1775	1951	90.98%
Upper Unburned	7	355	638	993	64.25%
Unsterilized Native	26	294	4175	4469	93.42%
Upper Unburned	7	115	1541	1656	93.06%
Upper Burned	10	129	1351	1480	91.28%
Lower Burned	9	50	1283	1333	96.25%
Potting mix	8	793	21	814	2.58%

Survival was higher (90-100%) for bioassay seedlings grown in burned soils regardless of sterilization than for seedlings grown in unburned soils (70%) (Table 3.3). However, a comparison of mortality rates among all bioassay treatments did not reveal a significant difference (p-value = 0.33) possibly due to small sample sizes. If survival rates for unsterilized and sterilized soils are combined, then seedlings growing in burned soil have significantly higher survival than those in unburned soils. (p-value = 0.02).

Six morphotypes were initially found in the bioassay study (Table 3.4). Fingerprint analysis of the ITS region revealed hidden diversity occurring in the E-strain morphotype as three taxa of *Wilcoxina* (*W. mikolae, W. rehmii, Wilcoxina* sp.1). Three other morphotypes were determined to be suilloid species by fingerprint analysis, one was determined to be a suilloid by morphological characters due to lack of molecular information, and one was left as an unknown due to lack of molecular information and definitive morphological characters.

Table 3.3: Frequency and abundance of native and nursery ectomycorrhizae (ECM) on whitebark pine bioassay seedlings in various soil treatments. The potting soil was MSU Sunshine mix that was not sterilized. Native soil was collected from burned and unburned whitebark pine forests in the Gallatin National Forest, MT. Native fungi are primarily suilloids and putative nursery fungi are E-strain.

	Upper Unburned Unsterilized	Upper Unburned Sterilized	Upper Burned Unsterilized	Upper Burned Sterilized	Lower Burned Unsterilized	Potting soil Unsterlized
% seedling survivial	70	70	100	100	90	80
Frequency of greenhouse fungi (%)	57	100	100	90	100	10
Frequency of native fungi (%)	57	0	10	0	44	0
Relative abundance of greenhouse fungi (%)	64	100	91	100	85	100
Relative abundance of native fungi (%)	36	0	9	0	15	0
Species richness	3	1	2	1	5	1

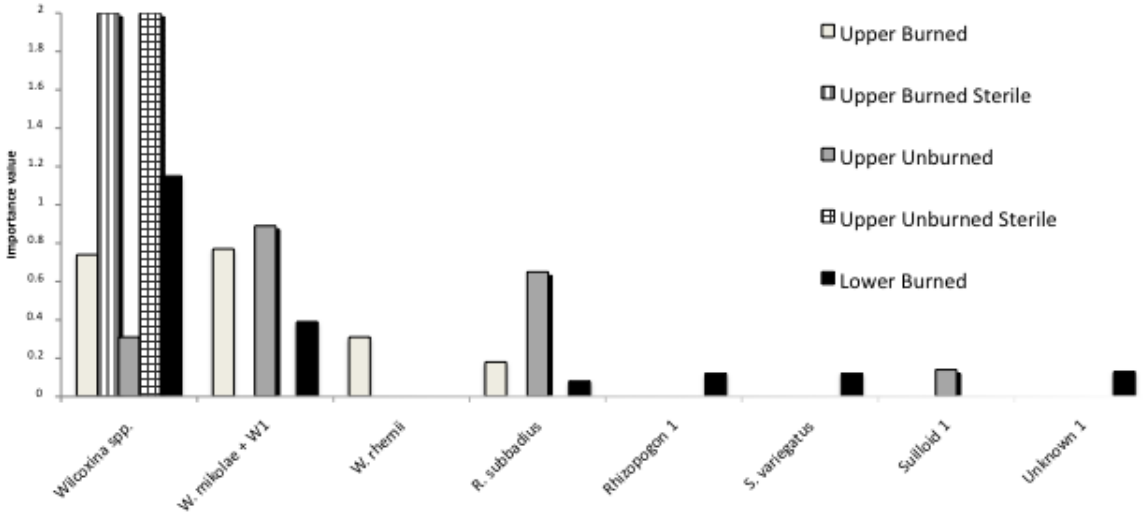
Table 3.4: Taxonomic matches for morphotypes of ECM in the bioassay of whitebark pine seedlings using NCBI Blast search.

Study Name	Best Match	% Identity	Overlap
Wilcoxina mikolae	Wilcoxina mikolae DQ069000	98%	579/587
Wilcoxina rhemii	Wilcoxina rhemii AF266708	98%	510/520
Wilcoxina 1	Wilcoxina sp DQ150131.1	97%	572/589
Wilcoxina spp.	Morphology	NA	NA
Rhizopogon, Amylopogon clade	Rhizopogon subbadius AF377152	98%	748/762
Rhizopogon 1	Rhizopogon: many weak matches	76%	504/661
Suillus variegatus	Suillus variegatus AY898622	98%	706/719
Suilloid 1	No sequence data, morphology	NA	NA
Unknown1	No sequence data, morphology	NA	NA

The importance values (Figure 3.2) highlight the dominance of *Wilcoxina* species for all treatments and the presence of a diversity of native suilloid fungi in the unsterilized native soils at low relative levels.

The most frequent and abundant taxon in all soil types was *Wilcoxina*, which occurred on 29 of the 51 surviving seedlings in all soil types. There were an average of 72 tips of *Wilcoxina* for all seedlings or an average of 126 tips for only those seedlings on which it occurred (Appendix C). The other group of fungi represented in more than a single sample was the suilloid clade (*Rhizopogon* and *Suillus*). Of these, the *Rhizopogon subbadius* clade has the highest relative frequency and abundance, occurring only in the unsterilized native soils.

Figure 3.2: Importance values (relative frequency plus relative abundance) for ECM taxa found on whitebark pine bioassay seedlings by soil type. Native soil was collected from burned and unburned whitebark pine forests, Gallatin National Forest, MT.



The mean diversity measures for all treatments in the bioassay are low (Table 3.5). This is due to many individual trees having only one or no associated

ectomycorrhizal taxa. The diversity for all controls was zero as they only hosted the E-strain fungus. An analysis of variance (ANOVA) found no significant difference in the diversity of ectomycorrhizal fungi among the bioassay treatments, due to the large variances.

Table 3.5: Mean Shannon's Diversity values of ECM fungi on roots in bioassay treatments (unsterilized) compared with results for field seedlings.

Soil Type	Seedling State	Mean Diversity	Standard Error	Standard Deviation
Upper unburned	Bioassay	0.19	0.1	0.27
Upper burned	Bioassay	0.04	0.04	0.13
Lower burned	Bioassay	0.18	0.09	0.28
Upper unburned	Native Field	0.56	0.04	0.29
Upper burned	Native Field	0.32	0.08	0.38
Upper burned	Planted Field	0.21	0.04	0.33

The diversity of ectomycorrhizal fungi on bioassay seedlings was compared to results from seedlings growing on the field sites. Comparisons were made of 1) bioassay seedlings planted in unsterilized native burned soil, 2) bioassay seedlings planted in unsterilized unburned native soil, 3) nursery seedlings planted on the burn, and 4) seedlings naturally regenerating in the unburned whitebark pine forest. Students t-tests found that seedlings in the field were significantly more diverse in ectomycorrhizal fungi than their greenhouse counterparts (p-value for burned soil = 0.004, p-value for unburned soil = 0.01).

A comparison of Importance Values for ectomycorrhizal fungi found on roots of seedlings in the greenhouse bioassay and those on field seedlings revealed differences. Important ectomycorrhizal species found on field seedlings are missing from both the burned and unburned soil treatments in the greenhouse bioassay (Figure 3.3 & 3.4). Four taxa that are both frequent and abundant on seedlings planted in the burn are missing in

the greenhouse bioassay (*Pseudotomentella nigra, Amphinema byssiodes, Cenococcum geophilum,* Thelephoroid species).

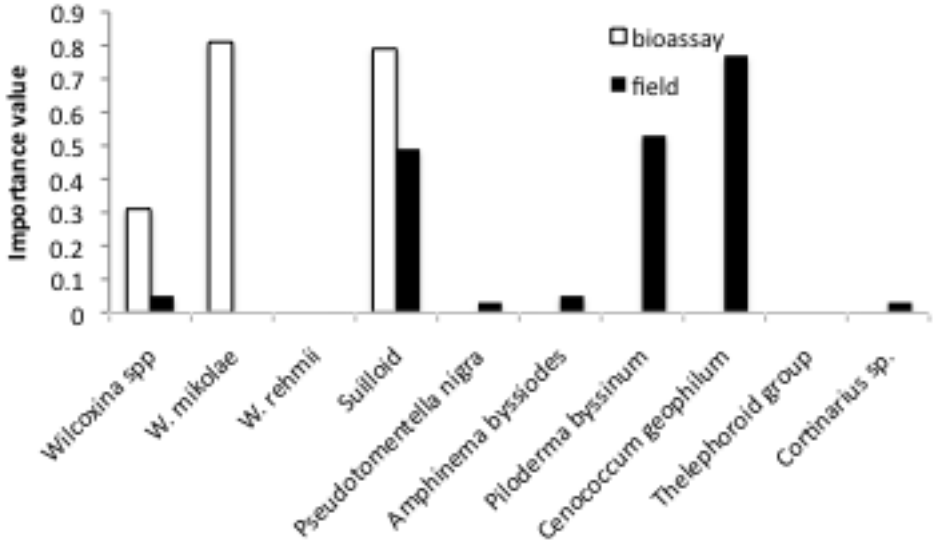


Figure 3.3: Comparison of importance values for major ectomycorrhizal taxa on whitebark pine seedlings a) out-planted in a burn (field) and b) grown in soil from the burned area in greenhouse conditions (bioassay seedlings).

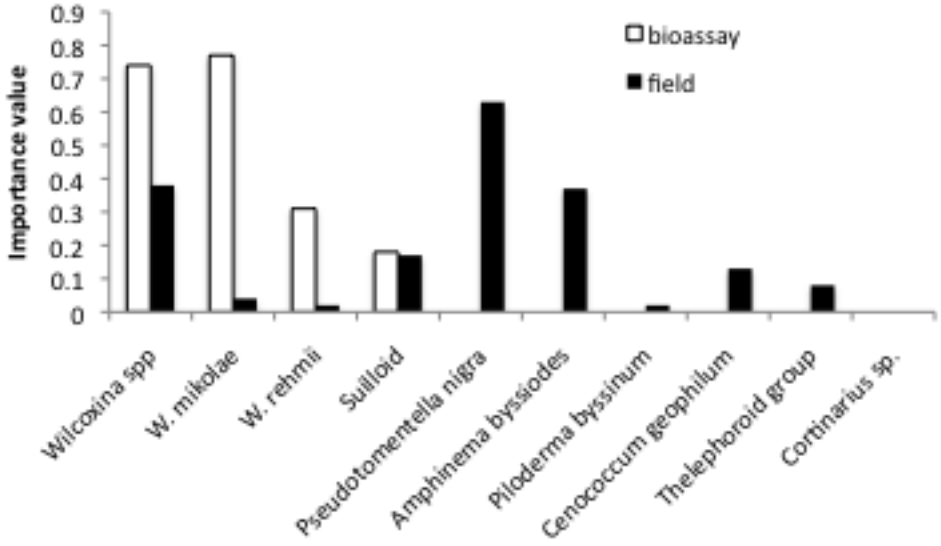


Figure 3.4: Comparison of importance values of major ectomycorrhizal taxa between whitebark pine seedlings a) naturally regenerating in an unburned forest (field) and b) grown in soil from the unburned area in greenhouse conditions (bioassay seedlings).

Similarly, two species that are both frequent and abundant on seedlings naturally regenerating in the unburned area are also missing in the greenhouse bioassay (*Cenococcum geophilum, Piloderma byssinum*). E-strain species are dominant in the greenhouse with values two times higher than for field seedlings in burned soil and ten times higher than field seedlings regenerating naturally on unburned soil in the intact forest.

Discussion

Ectomycorrhizal Fungi in Soil as Revealed by Greenhouse Bioassays:

A large percentage of the 200,000 whitebark pine seedlings planted in the last two decades have died. A project to monitor the survival of whitebark pine seedlings throughout their range found that the mean survival rate for 36 sites representing 114,677 seedlings was 42% with survival decreasing over time (Izlar 2007). While many abiotic factors could be responsible for the large die-offs (drought, temperature, soil type, etc.), biotic factors such as microsite, micro-organism communities and timely mycorrhization of seedlings could also play a role.

The greenhouse bioassay showed that suilloid fungi (*Rhizopogon, Suillus*) were present in the soil of the Fridley Burn (both upper and lower restoration areas) and in the adjacent unburned whitebark pine forest, with a higher diversity in the latter. In fact, nearly half of the ectomycorrhizal fungal species diversity discovered in the bioassay consists of *Rhizopogon* and *Suillus* species. This is of importance because suilloids have been found to be critical in the regeneration of other pine species (Kjøller and Bruns

2003). Most of these taxa show host specificity on some level; some are restricted to pines, others to 5-needle or 2-needle pines, and others to stone pines that include whitebark pine (Bruns et al. 2002, Mohatt and Cripps 2005). The presence of *Rhizopogon* on planted and natural seedlings indicates that these potentially vital fungi have not been lost in the burn or have been brought in by vectors (four years since fire) and are able to form mycorrhizae on planted nursery-propagated seedlings. However, suilloid fungi were not dominant on the field seedlings and were recorded in low levels. Results from the bioassay seedlings can at least indicate to managers that suilloids are present and not lacking in the soil. Their low levels five years after the burn could be a concern and future monitoring could be necessary over time.

To date, 12 species of suilloids have been recorded with *Pinus albicaulis* in the Greater Yellowstone area (Mohatt et al. 2008, Cripps and Trusty 2007) and most appear to be specific for 5-neeedle pines and some for stone pines. At least two of the taxa reported from the bioassay (*S. variegatus, R. subbadius* clade) are reported from whitebark pine forests in other areas of the Greater Yellowstone Ecosystem (Mohatt et al. 2008).

The remainder of the taxa discovered in the bioassay consist of strains or species of *Wilcoxina* also called E-strain. Some species of *Wilcoxina* are considered to be "nursery fungi" proliferating in this setting. A majority of seedlings in the bioassay, in both burned and unburned soil hosted at least one type of *Wilcoxina*. All seedlings planted in the sterilized soil hosted E-strain, showing that at least one strain originated

from the seedlings themselves, likely because they were first raised for 2-3 years under nursery conditions.

There appears to be some disagreement in the literature as to the ecology of *Wilcoxina* species. *Wilcoxina mikolae* has been found on *Pinus sylvestris* (Iwanski and Rudawska 2007) and *Picea abies* (Trocha et al. 2006) in nurseries in Poland, and it is found in U.S. nurseries as well. Several U.S. studies found *W. mikolae* to be common on field seedlings after burns (Yang & Korf 1985, Egger et al. 1991, Baar et al.1999, Mah et al. 2001). Examination of the ectomycorrhizal colonization of *Pinus muricata* seedlings after a stand-replacing burn in California found *W. mikolae* to be the second most abundant ectomycorrhizal fungus on naturally regenerating field seedlings and it was also reported on bioassay seedlings (Baar et al. 1999). However, Fujimura et al. (2005) reported *Wilcoxina rehmii* to be the fungus present after fire in the ponderosa pine forests of eastern Oregon. The current whitebark pine study revealed three types of *Wilcoxina*, including both *W. mikolae* and *W. rehmii* on seedlings. It was confirmed that *Wilcoxina mikolae* can originate in the greenhouse as its fruiting bodies were found with nursery seedlings. *Wilcoxina mikolae* also occurred on naturally regenerating whitebark pine seedlings in the field. *Wilcoxina rehmii* was only found on bioassay seedlings growing in unsterilized burned soil. While some *Wilcoxina* mycorrhizae were identified through molecular techniques, many were identified solely through morphological characters; therefore it is difficult to assess the species proportions for this genus with accuracy. We do know that *W. mikolae* occurred on seedlings before out-planting but it is unclear if it persisted or was replaced by native strains.

It is possible that species of *Wilcoxina* are not restricted to any specific setting but are common in areas of low competition such as nurseries or after disturbances such as fires (Fujimura et al. 2005, Avis and Charvat 2005).

Quantitative Examination of ECM Fungi in Bioassays for Burned and Unburned Soil:

The results of the greenhouse bioassay show a high level of mycorrhizal colonization on all bioassay seedling treatments, including those with sterilized soil with one exception. The low ectomycorrhizal colonization for the bioassay seedlings in the potting soil is hypothesized to be the result of the anti-fungal properties of the Montana State University (MSU) Sunshine Potting Mix (Mahony 2005). While this problem may be specific to MSU, the fungal suppressive properties of soils should be considered in this type of research.

There were high levels of ectomycorrhizal colonization for most bioassay treatments; however, seedlings grown in sterilized soil hosted only E-strain fungus. This confirms that the soil sterilization process was effective in removing inoculum from the field soil in the controls and that at least one E-strain species was likely on the seedlings before they were transplanted to the field soils, or that the fungus proliferates in the nursery setting.

E-strain makes up 65-90% of all mycorrhizal tips and is the most frequent colonizer on bioassay seedlings growing in both burned and unburned unsterilized soil. In another greenhouse bioassay of Yellowstone National Park soils, E-strain was also found to be highly abundant and frequent on whitebark pine seedlings from the same nursery

(Cripps and Trusty 2007). The level of E-strain colonization is similar to that found by Torres and Honrubia (1997) in a greenhouse bioassay of another pine (Table 3.6). They compared the ectomycorrhizal potential of burned and unburned soils from a *Pinus halepensis* forest in Spain. The most frequent and abundant ectomycorrhizal fungal taxa found in both burned and unburned bioassay soils in their research was E-strain (50-90%). This study started with seed, in contrast to the current one, which suggests that that E-strain thrives in greenhouse settings regardless of whether seeds or seedlings are used. More evidence for this comes from a greenhouse bioassay to determine the ectomycorrhizal fungal associated with *Pinus contorta* in three soils from Oregon coastal sand dunes (Ashkannejhad and Horton 2006). In their study the average frequency of E-strain was five to ten times higher in the greenhouse setting as compared to the field.

Over the three different soil types, E-strain had the highest mean frequency of any taxa in the greenhouse bioassay. Species of E-strain are common in almost all greenhouse bioassays using pine species as the trap organism. Izzo et al. (2006a) applied different heat treatments to soil from the Sierra Nevada mountains and found that E-strain had the highest frequency across all treatments but one. In a greenhouse bioassay examining the ectomycorrhizal inoculum potential of soils near *Pinus longaeva*, E-strain was the third most abundant taxa (Bidartondo et al. 2001). An important unanswered question is if it prevents colonization by native fungi in the field.

The suilloid fungi (*Rhizopogon* and *Suillus* species) were the only other taxonomic group found in the greenhouse bioassay. These fungi were only on bioassay seedlings growing in unsterilized native soil and overall they had a lower relative

frequency and abundance than the E-strain fungi. Suilloid fungi have been commonly picked up in other greenhouse bioassays. The bioassay by Torres and Honrubia (1997) found suilloid fungi to be the second most frequent group after E-strain, as reported in the present study. In their work with pines on Oregon sand dunes, Ashkannejhad and Horton (2006), found suilloids (as a group) to have the greatest average frequency, with *Rhizopogon fuscorubens* and E-strain tied for the highest individual mean frequency. In a greenhouse bioassay with pines using soil from the Sierra Nevada mountains exposed to different heat treatments, suilloids had the highest or second highest frequency (Izzo et al. 2006a). Suilloids were the second most abundant group in a bioassay of soils near *Pinus longaeva* trees (Bidartondo et al. 2001). Another greenhouse bioassay found that suilloid fungi had the highest frequency in soil from a *Pinus muricata* stand following a severe fire (Baar et al. 1999). The dominance of suilloids (next to E-strain) for the studies reviewed in table 3.6 suggests there may be a bias towards suilloids as regards native fungi in bioassays, regardless of methods. While some studies suggest that the bioassay is reflective of field results, this may not be true in all cases

Comparison of Bioassay Results to Communities
of Ectomycorhizal Fungi on Seedlings in the Field:

Seedlings in the field, whether naturally regenerating or planted, had higher species richness than bioassay seedlings in the greenhouse. This was true for both burned (1.1 vs 1.7) and unburned (1.7 vs 2.2) soils. The overall species richness of the bioassay seedlings was also much lower than that of field seedlings.

Table 3.6: Review of bioassays that use pine species as the trap organism for ectomycorrhizal fungi. The "# of spp Field" = ECM fungal taxa on seedlings growing naturally *in situ*; "# spp Bio" = ECM fungal taxa on bioassay seedlings in field soils in a nursery setting; "Overlap" = ECM fungi found on both bioassay and seedlings *in situ*; "Bio Suilloids" = ECM fungi on bioassay seedlings in the suilloid group; "Initial" = initial status of trap tree species; "Soil Bioassay Mixture" = proportions in bioassay medium.

Tree Species	# spp Field	# spp Bio	Overlap	Bio Suilloids	Initial	Soil Bioassay Mixture	Reference
Pinus muricata	7	8	4	3[1]	seed	soil/sand (1:1); 1/2, 1/20, 1/200	Baar et al. 1999
Pinus contorta: mature	25	18	10	9	seed	soil/peat/vermiculite (1:1:1)	Ashkannejhad & Horton 2006
Pinus contorta: deflation	21	18	9	9	seed	soil/peat/vermiculite (1:1:1)	Ashkannejhad & Horton 2006
Pinus contorta: isolated	10	10	4	6	seed	soil/peat/vermiculite (1:1:1)	Ashkannejhad & Horton 2006
Pinus longaeva	8 (1sample)	10	4?	1[2]	seed	soil	Bidartondo et al. 2001
Pinus halpensis: burned	NA	5	NA	2[2]	seed	soil/vermiculite (1:1)	Torres & Honrubia 1997
Pinus halpensis: unburned	NA	7	NA	2[2]	seed	soil/vermiculite (1:1)	Torres & Honrubia 1997
Pinus jeffreyi	NA	6	NA	3[3]	seed	soil/sand (1:1)	Izzo et al. 2006a
Pinus edulis	NA	18 (6 known)	NA	2[4]	germinant	soil	Haskins & Gerhing 2005
Pinus albicaulis: replaced	NA	3	NA	2[2]	seedling	soil/sand/peat (2:1:1)	Cripps & Trusty 2007
Pinus albicaulis: native	7	4	3	3[2]	seedling	soil/sand/peat (2:1:1)	Cripps & Trusty 2007
Pinus albicaulis: burned	18	5	4	1[2]	seedling	soil/sand/peat (2:1:1)	Trusty & Cripps
Pinus albicaulis: unburned	14	2	2	1[2]	seedling	soil/sand/peat (2:1:1)	Trusty & Cripps

[1] 74% of all tips were suilloid fungi.
[2] Suilloid fungi are the most abundant and frequent of all Basidiomycota fungi found.
[3] 40% of all fungi were suilloids and 100% of all Basidiomycota were suilloid fungi.
[4] 25% of all fungi found within the pinyon pine zone were suilloid fungi.

Bioassay seedlings in burned soil hosted a total of five taxa whereas the field seedlings hosted 22 taxa. Similarly, for the seedlings in the unburned soil, the bioassay detected two taxa and the field study found 14. Only a few studies have compared greenhouse bioassay results to those found on field seedlings. One study made this comparison for soils from three adjacent habitat types in Oregon coastal sand dunes (Ashkannejhad and Horton 2006). Seedlings growing in these three habitat types had higher species richness than seedlings in the greenhouse bioassay, although numbers were still high in the bioassay likely due to the sensitivity of the method used.

In the present study, many dominant taxa associated with the field seedlings in both unburned and burned forests were not found by the bioassay. *Wilcoxina* species and suilloid fungi are the most frequent and abundant groups for both unburned and burned bioassay seedlings. One difference was that *Wilcoxina* species had a higher importance value (high relative frequency and relative abundance) on field seedlings in the burn, compared to that for seedlings in the unburned area.

While the suilloid taxa were actually found in similar relative amounts on bioassay and field seedlings, the other major taxa that occurred on field seedlings were completely missing from the greenhouse bioassay. The fungal taxa with the highest importance values in the field (*Pseudotomentella nigra* and *Amphinema byssiodes*) for the burned area and (*Cenococcum geophilum* and *Piloderma byssinum*) for the unburned area were not found by the bioassay. In their study of ectomycorrhizal communities in different habitat types in Oregon sand dunes, Ashkannejhad and Horton (2006) found between 4 and 15 types for each habitat type in the field that did not appear in the

bioassay. However, their bioassay also revealed taxa that were not found on the field seedlings. A study that examined the ectomycorrhizal fungal communities on seedlings growing on a burned *Pinus muricata* forest and those on that soil in a greenhouse bioassay found three distinct fungal taxa on the field seedlings that were not detected in the bioassay (Baar et al. 1999). In addition, research on the inoculum potential of soils near *Pinus longaeva* in California found four ectomycorrhizal taxa in the field (with a single sample) not found in the greenhouse bioassay (Bidartondo et al. 2001).

There are multiple possibilities as to why the major fungi on seedlings in the field study were not detected by the bioassay while the suilloids were usually revealed, at least in low amounts. Reasons for the missing taxa might be explained by 1) the long-lived propagules of suilloids, 2) loss of viable inoculum of certain species, 3) timing of the study, 4) and/or a possible competitive advantage for suilloids and E-strain in bioassay conditions. Each possibility is discussed in turn.

One possible explanation for the constant appearance of suilloid fungi in the bioassay is that they appear to have long lived propagules while many other ectomycorrhizal taxa do not. Ectomycorrhizae in mature forest systems often form from root contact with mycelium of existing fungal species (Taylor and Bruns 1999, Grogan et al. 2000). In a comparison of the ectomycorrhizal fungal community in a mature *Pinus muricata* forest with that of the resistant propagule community in the soil, Taylor and Bruns (1999) found that individual sample cores within the mature forest often had an overabundance of single species, suggesting growth by mycelial contact. This would result in a high importance value (many ectomycorrhizal tips counted) from one initial

contact with a species. It is possible that the whitebark pine bioassay was initiated too long after the fire to adequately capture short-lived propagules such as those that form mainly by direct contact. Research has shown that in clear-cut areas, which are similar to burns in that all mature trees have been killed, most ectomycorrhizal fungi have a short survival time in the soil. Wiensczyk et al. (2002) reported that "In areas that have been clear-cut logged, ectomycorrhizal roots do not typically survive more than two years". In addition, research in Montana found that mycorrhizal potential decreased drastically six months after logging in a mixed spruce/fir stand (Harvey et al. 1979).

It may be that only those ectomycorrhizal fungi with propagules able to maintain viability for long periods were available to inoculate the bioassay seedlings. Suilloid fungi have propagules that can maintain viability for long periods of time (Baar et al. 1999, Kjøller and Bruns 2003, Ashkannejhad and Horton 2006, Izzo et al. 2006a). In a study of the *Rhizopogon* spore bank communities in five forests in California, Kjøller and Bruns (2003) assumed a large uniform distribution of *Rhizopogon* spores. The assumed high number of infective propagules spread evenly in the forest soils was in part attributed to the longevity of suilloid spores. Their reasoning was that *Rhizopogon* fruiting-bodies are hypogeous, which typically results in a patchy distribution of spores in comparison to wind dispersal, which results in a more even spread of spores. They determined that the patchiness could have been overcome in time if the spores were long lived and moved around in processes that move soil. In our methodology the soil was collected four years after the out-planting of seedlings in the field, and it could be that suilloid fungi were the main propagules still viable to inoculate bioassay seedlings.

If a loss of viable inoculum is the reason for missing the main taxa on bioassay seedlings, one would expect that naturally regenerating seedlings growing on the burn would mainly be colonized with suilloid fungi, as the naturally regenerating seedlings are generally younger than those planted directly following the fire. However, this was not found to be the case. The naturally regenerating seedlings on the burn were ectomycorrhizal with *Rhizopogon* species, but the dominant mycorrhizal taxa were still *Amphinema* and *Pseudotomentella* (the same as the planted seedlings). It is possible that there is a critical point at which the inoculum source for certain ectomycorrhizal is no longer viable and that this point occurred after the natural seedlings germinated but before soil was collected for bioassay, but this seems unlikely.

The nursery-grown seedlings, which were part of the restoration effort after the fire, were out-planted less than one year after the burn. This could have possibly allowed them to pick up major taxa that were missing from the bioassay before the soil lost its inoculum potential over the next three years, but *Amphinema* and *Pseudotomentella* were dominant species in the burn for both planted and natural seedlings. However, there was no such disturbance in the intact mature forest that would have caused a loss of inoculum potential and the main ectomycorrhizal fungal taxa from the mature forest (*Piloderma* and *Cenococcum*) were also missing from the bioassay soil. This points to some other reason for the dominance of suilloid ectomycorrhizae and the lack of other main taxa in the bioassay.

It is also possible that misrepresentation of the ectomycorrhizal community could be caused by the use of seedlings rather than seeds for the bioassay. However, previous

bioassays that used seeds still found the majority of the taxa to be suilloids (Baar et al. 1999, Bidartondo et al. 2001, Ashkannejhad and Horton 2006). After a stand-replacing wildfire in a *Pinus muricata* forest in California, Baar et al. (1999) found that 73.4% of the ectomycorrhizal species composition was *Rhizopogon* in a bioassay that used seeds. In addition, an unpublished study using 100% field soil from the same West Pine Creek Research Site (Chapter 2) with whitebark pine seeds as the bioassay organism again found suilloids to dominant in the bioassay (Cripps and Johnson unpublished).

Possibly, the preponderance of suilloid fungi, and the lack of other major taxa found in the field, is a result of suilloid fungi having a competitive advantage in colonization of pine in nurseries. The suilloid genus *Rhizopogon* is known to grow in nursery settings (El Karkouri et al. 2002, El Karkouri et al. 2004, El Karkouri et al. 2005). It is possible that conditions in the nursery setting are an advantage for suilloids, allowing them to outcompete/exclude other fungi and bias results. Since suilloids occur in nature and in the greenhouse in similar frequencies and abundances, there is no evidence to suggest they are more or less competitive in nature.

It has been suggested that *Rhizopogon* propagules can make up a vast majority of the resistant propagule community (Kjøller and Bruns 2003, Taylor and Bruns 1999, Baar et al. 1999, Izzo et al. 2006a, Rusca et al. 2006). Kjøller and Bruns (2003). A bioassay study of *Rhizopogon* communities in California found the (assumed) abundance of *Rhizopogon* spores to be large enough so that the make-up of the ectomycorrhizal community was not impacted by distances of one kilometer or a 50-fold dilution of the

soil. Methods used for the bioassay can also affect the outcome and this is discussed in the next section.

Evaluation of Bioassay Method:

There are multiple ways to potentially improve the results of the bioassay: 1) timing of soil collection, 2) soil dilution, 3) soil storage (including drying), 4) and use of various soil amendments (sand, peat, vermiculite). One possibility would be to collect soil and carry out the bioassay soon after the fire. This might increase the chance of the propagules still being viable. Alternatively, they may be lost due to fire.

Dilution of the field soil with various amendments can also affect bioassay results. Baar et al. (1999) showed that too little dilution can result in *Rhizopogon* propagules swamping out other potential species and too much dilution will also result in *Rhizopogon* propagules functioning as the only species present. However, it could be assumed that if there were more viable *Rhizopogon* propagules than those of other species, they would also be dominant on planted seedlings in the field as well as on the bioassay seedlings, which was not the case in this study. Also, the methodology for this study of pooling individual soil samples for both the burned and unburned areas may have benefited *Rhizopogon* species, since combination would distribute any *Rhizopogon* propagules even if the system is patchy. Not mixing soil samples might improve the sensitivity of the bioassay.

Baar et al. (1999) found that drying bioassay soil had significant impacts on the types and frequencies of ectomycorrhizal fungi that were able to colonize bioassay

seedlings. Drying the soil resulted in changes of frequency for multiple ectomycorrhizal species and changes in the species richness of soil profiles.

Starting from seed might remove some of the ambiguity of the source of the ectomycorrhizal fungi, such as E-strain found on bioassay seedlings at least initially. Finally, the inoculation of missing species onto greenhouse seedlings might confirm their ability to grow under greenhouse conditions in a particular soil type. Bioassay results might also reflect a combination of all the concerns discussed.

Conclusion:

An important consideration for restoration strategies where whitebark pine is planted in a burn is the potential for timely mycorrhization of seedlings. Conditions appeared optimal at the West Pine Creek Restoration Site: seedlings were planted within one year of the burn (fungal propagules possibly still present), the burn was adjacent to an intact unburned whitebark pine forest (a potential inoculum source) and small and large mammals traversed both areas (possible spore dispersal vectors). The latter could be important since mammals such as squirrels and deer have been shown to spread the spores of both the underground fungus *Rhizopogon* as well as those of *Suillus* (Ashkannejhad and Horton 2006) and mammals were common on the burned area. In addition to their mycorrhizal function, the suilloid fungi are also an important food source for some mammals at particular times of the year (Mattson et al. 2001). This may be another important consideration in restoration strategies.

Suilloid fungi do appear to be present in the soil on the Fridley Burn according to the bioassay and field results, although they were at low levels on all field seedlings. This

brings up the question of what factors might promote or delay colonization of important native fungi such as the suilloids.

The use of commercial mycorrhizal inoculum should not be considered as an alternative. It typically contains non-native fungi that are not specific to whitebark pine which have the potential to upset the delicate balance of these complex and sensitive ecosystems. Commercial inoculum does not contain fungi native to whitebark pine and may also promote competitive tree species. In some cases, fungi in commercial products may be considered exotics, especially in national parks. Mammals which depend on particular species for food might also be affected.

It is important to note that while conditions appeared to be optimized for the colonization of whitebark pine seedlings with appropriate mycorrhizal fungi, survival of the seedlings out-planted at the West Pine Creek Restoration Site was low (22-42%). This leaves room for direct investigations into the ability of ectomycorrhizal fungi inoculated onto whitebark pine seedlings prior to out-planting to increase the survival of planted whitebark pine seedlings on this type of restoration site.

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APPENDICES

APPENDIX A

SOIL TEMPERATURES IN BURNED AND UNBURNED WHITEBARK PINE FORESTS IN THE WEST PINE CREEK RESTORATION AREA

Appendix A: Temperatures (^{o}C) of burned and unburned soil in the West Pine Creek Restoration area taken over four days at three distances (0,100,200 meters) on all transects in 2006.

Transect	Burned Soil			Unburned Soil		
July 12, 2006	0 m	100 m	200 m	0 m	100 m	200 m
1	22	19	19	14	13	16
2	20	21	23	15	17	13
3	18	19	21	14	15	15
July 17, 2006						
1	27	22	22	16	18	16
2	25	26	24	14	20	15
3	26	25	22	14	17	17
July 29, 2006						
1	25	26	25	18	18	17
2	26	22	24	17	21	20
3	27	25	26	15	16	17
August 8, 2006						
1	24	22	20	18	15	15
2	24	16	19	12	18	16
3	31	33	23	14	16	15

APPENDIX B

ECTOMYCORRHIZAL COMMUNITIES ON SEEDLINGS THAT WERE PLANTED OR NATURALLY REGENERATING IN A BURNED AND UNBURNED WHITEBARK PINE FOREST

Appendix B: Individual whitebark pine tree sampled, their planting status, fire status, the ectomycorrhizal fungi associated with them, and tip abundance of ectomycorrhizal fungi. A = *Amphenima*, C = *Cenococcum*, NA = No species, P = *Pseudotomentella*/*Piloderma*, R = *Rhizopogon*,S = *Suillus*, W = *Wilcoxina*.

TreeID	Planting Status	Fire Status	ECM Species: Abundance
UU1	Natural	Unburned	P. nigra:14, Unknown type:10
UU2	Natural	Unburned	C. geophilum:16, R. roseolus:128
UU3	Natural	Unburned	Suilloid spp.:46
UU4	Natural	Unburned	C. geophilum:21, P. byssinum:14, Suilloid spp.:27
UU5	Natural	Unburned	C. geophilum:12, P. byssinum:4
UU6	Natural	Unburned	C. geophilum:11, P. byssinum:56, Suilloid spp.:10
UU7	Natural	Unburned	C. geophilum:21, P. byssinum:44
UU8	Natural	Unburned	C. geophilum:36, P. byssinum:50
UU9	Natural	Unburned	C. geophilum:11, P. byssinum:9
UU10	Natural	Unburned	C. geophilum:60, P. byssinum:53
UU11	Natural	Unburned	Wilcoxina spp.:9, C. geophilum:51, P. byssinum:38, Species 2:16
UU12	Natural	Unburned	C. geophilum:5, P. byssinum:26
UU13	Natural	Unburned	P. byssinum:23
UU14	Natural	Unburned	C. geophilum:24
UU15	Natural	Unburned	Wilcoxina spp.:11, C. geophilum:2, P. byssinum:27
UU16	Natural	Unburned	C. geophilum:28, R. roseolus:93
UU17	Natural	Unburned	C. geophilum:12, Suilloid spp.:19
UU18	Natural	Unburned	C. geophilum:17, P. byssinum:80
UU19	Natural	Unburned	C. geophilum:19, P. byssinum:6
UU20	Natural	Unburned	C. geophilum:19, Suilloid spp.:20
UU21	Natural	Unburned	C. geophilum:8, Suilloid spp.:60
UU22	Natural	Unburned	P. nigra:8, C. geophilum:6, P. byssinum:4
UU23	Natural	Unburned	C. geophilum:15, P. byssinum:12, R. roseolus:41
UU24	Natural	Unburned	C. geophilum:22, P. byssinum:2, Cortinarius:11
UU25	Natural	Unburned	C. geophilum:3, P. byssinum:15, Unknown type:35
UU26	Natural	Unburned	A. byssoides:89, C. geophilum:13, R. roseolus:14
UU27	Natural	Unburned	Wilcoxina spp.:14, P. byssinum:4
UU28	Natural	Unburned	Rhizopogon spp.:43
UU29	Natural	Unburned	A. byssoides:18, P. byssinum:65
UU30	Natural	Unburned	C. geophilum:59, Suilloid spp.:3
UU31	Natural	Unburned	Wilcoxina spp.:53: Suilloid spp.:3
UU32	Natural	Unburned	P. byssinum:39, Russula sp.:23
UU33	Natural	Unburned	C. geophilum:30, P. byssinum:16
UU34	Natural	Unburned	C. geophilum:37, P. byssinum:13
UU35	Natural	Unburned	C. geophilum:28, Unknown type:30
UU36	Natural	Unburned	C. geophilum:88, Unknown type:19
UU37	Natural	Unburned	C. geophilum:35, Suilloid spp.:139
UU38	Natural	Unburned	P. nigra:33, C. geophilum:4, R. subbadius:143
UU39	Natural	Unburned	C. geophilum:18, P. byssinum:21
UU40	Natural	Unburned	C. geophilum:18, Rhizopogon spp.:12
UU41	Natural	Unburned	C. geophilum:26
UU42	Natural	Unburned	C. geophilum:16
UU43	Natural	Unburned	C. geophilum:69, P. byssinum:126, S. variegatus:30
UU44	Natural	Unburned	C. geophilum:15, P. byssinum:6, S. placidus:15
UU45	Natural	Unburned	C. geophilum:36, P. byssinum:34
UU46	Natural	Unburned	C. geophilum:46, P. byssinum:13
UU47	Natural	Unburned	P. byssinum:27, Unknown type:50
UU48	Natural	Unburned	C. geophilum:25, R. subbadius:27
UU49	Natural	Unburned	C. geophilum:61, Suilloid spp.:39
UU50	Natural	Unburned	C. geophilum:56, R. roseolus:24
UU51	Natural	Unburned	C. geophilum:6, P. byssinum:16, R. subbadius:16
UU52	Natural	Unburned	C. geophilum:10, P. byssinum:67, R. subbadius:19
UU53	Natural	Unburned	C. geophilum:22
UU54	Natural	Unburned	C. geophilum:46, S. variegatus:25
UU55	Natural	Unburned	C. geophilum:25, P. byssinum:37

Appendix B Continued

UU56	Natural	Unburned	C. geophilum:13, P. byssinum:23, R. roseolus:28
UU57	Natural	Unburned	C. geophilum:28, P. byssinum:2, Cortinarius:44
UU58	Natural	Unburned	C. geophilum:8, P. byssinum:3
UU59	Natural	Unburned	C. geophilum:7, P. byssinum:13
UU60	Natural	Unburned	C. geophilum:25, P. byssinum:9
NB1	Natural	Burned	Wilcoxina spp.:24
NB2	Natural	Burned	P. nigra:66
NB3	Natural	Burned	C. geophilum:17, R. roseolus:19, R. subbadius:47
NB4	Natural	Burned	NA
NB5	Natural	Burned	P. nigra:2
NB6	Natural	Burned	W.mikolae:15, C. geophilum:2
NB7	Natural	Burned	NA
NB8	Natural	Burned	Wilcoxina spp.:24
NB9	Natural	Burned	P. nigra:26, C. geophilum:13, R. subbadius:21
NB10	Natural	Burned	P. nigra:14, C. geophilum:4
NB11	Natural	Burned	P. nigra:17, Phialocephala fortinii:20
NB12	Natural	Burned	Coltricia sp.:74
NB13	Natural	Burned	Wilcoxina spp.:34, C. geophilum:1
NB14	Natural	Burned	Wilcoxina spp.:26, Rhizopogon spp.:21
NB15	Natural	Burned	W.mikolae:25, A. byssoides:12
NB16	Natural	Burned	A. byssoides:94
NB17	Natural	Burned	C. geophilum:18
NB18	Natural	Burned	Wilcoxina spp.:21, C. geophilum:1
NB19	Natural	Burned	Wilcoxina spp.:37
NB20	Natural	Burned	Wilcoxina spp.:115, P. nigra:27, C. geophilum:1
NB21	Natural	Burned	Wilcoxina spp.:6, Unknown type:7
NB22	Natural	Burned	P. nigra:24, C. geophilum:12
NB23	Natural	Burned	Wilcoxina spp.:91
NB24	Natural	Burned	C. geophilum:7, P. byssinum:3
UB1	Planted	Burned	Tomentella sp.:12
UB2	Planted	Burned	C. geophilum:32, Unknown type:4
UB3	Planted	Burned	A. byssoides:52
UB4	Planted	Burned	A. byssoides:12
UB5	Planted	Burned	A. byssoides:36, Unknown type:4
UB6	Planted	Burned	A. byssoides:99
UB7	Planted	Burned	A. byssoides:23, P. nigra:2
UB8	Planted	Burned	A. byssoides:17, Unknown type:24
UB9	Planted	Burned	P. nigra:26
UB10	Planted	Burned	Thelephora sp.:80
UB11	Planted	Burned	Wilcoxina spp.:22
UB12	Planted	Burned	Thelephoroid:54
UB13	Planted	Burned	P. nigra:42
UB14	Planted	Burned	Species 3:91
UB15	Planted	Burned	A. byssoides:72
UB16	Planted	Burned	Wilcoxina spp.:5, A. byssoides:20
UB17	Planted	Burned	P. nigra:105
UB18	Planted	Burned	Wilcoxina spp.:131, C. geophilum:2,
UB19	Planted	Burned	A. byssoides:20
UB20	Planted	Burned	Wilcoxina spp.:11, C. geophilum:22
UB21	Planted	Burned	A. byssoides:11, Suilloid spp.:38
UB22	Planted	Burned	NA
UB23	Planted	Burned	Unknown type:24
UB24	Planted	Burned	Wilcoxina spp.:19, P. byssinum:61
UB25	Planted	Burned	Thelephora sp.:87
UB26	Planted	Burned	A. byssoides:21, C. geophilum:1, R. subbadius:48, Unknown type:8

Appendix B Continued

UB27	Planted	Burned	C. geophilum:4, P. nigra:197
UB28	Planted	Burned	A. byssoides:41
UB29	Planted	Burned	A. byssoides:44
UB30	Planted	Burned	W,rehmii:38, A. byssoides:22, Unknown type:32
UB31	Planted	Burned	Wilcoxina spp.:39
UB32	Planted	Burned	W.mikolae:73
UB33	Planted	Burned	Wilcoxina spp.:140
UB34	Planted	Burned	A. byssoides:17, C. geophilum:41
UB35	Planted	Burned	P. nigra:359
UB36	Planted	Burned	P. nigra:95, Suilloid spp.:14
UB37	Planted	Burned	Wilcoxina spp.:88
UB38	Planted	Burned	P. nigra:208
UB39	Planted	Burned	Wilcoxina spp.:84, P. nigra:108
UB40	Planted	Burned	P. nigra:105
UB41	Planted	Burned	Wilcoxina spp.:148, P. nigra:157, Rhizopogon spp.:55
UB42	Planted	Burned	Wilcoxina spp.:59, A. byssoides:14
UB43	Planted	Burned	Wilcoxina spp.:22, P. nigra:164
UB44	Planted	Burned	Wilcoxina spp.:34 , P. nigra:181, C. geophilum:4
UB45	Planted	Burned	A. byssoides:12, P. nigra:21, C. geophilum:21
UB46	Planted	Burned	Species 1:14
UB47	Planted	Burned	Suilloid spp.:27, Species 1:18
UB48	Planted	Burned	P. nigra:28, Unknown type:15
UB49	Planted	Burned	W.mikolae:44
UB50	Planted	Burned	Wilcoxina spp.:115
UB51	Planted	Burned	Unknown type:20
UB52	Planted	Burned	Wilcoxina spp.:7, Unknown type:48
UB53	Planted	Burned	Wilcoxina spp.:66, P. nigra:168
UB54	Planted	Burned	R. roseolus:22, Sebacinaceae:46
UB55	Planted	Burned	A. byssoides:143
UB56	Planted	Burned	W.mikolae:61
UB57	Planted	Burned	A. byssoides:17, Unknown type:15
UB58	Planted	Burned	P. nigra:180, Thelephoroid:53
UB59	Planted	Burned	A. byssoides:19, P. nigra:112, C. geophilum:5
UB60	Planted	Burned	P. nigra:271, R. subbadius:27

APPENDIX C

ABUNDANCES OF E-STRAIN AND SUILLOID FUNGI ON WHITEBARK PINE SEEDLINGS FOR DIFFERENT SOIL TYPES UNDER GREENHOUSE CONDITIONS

Appendix C: Abundance and frequency of E-strain and suilloid fungi on whitebark pine seedlings for all soil types growing under greenhouse conditions.

Tree #	Control		Upper Burned Non-Sterile		Upper Burned Sterile		Upper Unburned Non-Sterile		Upper Unburned Sterile		Lower Burned Non-Sterile	
	Suilloids	E-strain	Suilloids	E-strain	Suilloids	E-strain	Suilloids	E-strain	Suilloids	E-strain	Suilloids	E-strain
1	-	-	-	165	-	26	D	D	D	D	-	72
2	-	-	-	40	-	57	D	D	-	149	-	12
3	D	D	-	28	-	83	D	D	D	D	33	11
4	-	-	-	28	-	277	145	287	-	100	-	223
5	-	-	-	199	-	157	-	-	-	21	-	22
6	-	19	-	165	-	96	52	-	-	152	-	68
7	D	D	70	11	-	282	-	69	D	D	D	D
8	-	-	-	18	-	-	77	4	-	17	-	107
9	-	-	-	14	-	108	-	82	-	35	48	4
10	-	-	-	19	-	193	28	106	-	3	34	129
Freq	0%	10%	10%	100%	0%	90%	57%	71%	0%	100%	33%	100%