# Inoculation and Successful Colonization of Whitebark Pine Seedlings With Native Mycorrhizal Fungi Under Greenhouse Conditions

Paper

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**Abstract—**Efforts to maintain and restore whitebark pine (*Pinus albicaulis*) forests in western North America have increased dramatically over the last two decades and now include the planting of nursery-grown rust resistant seedlings in openings and burned areas. Over 200,000 nursery seedlings have been planted in the western U.S. but survival rates are low and in many areas approach zero. One possibility for enhancing seedling survival is application of mycorrhizal fungi in the greenhouse before out-planting. All pines require ectomycorrhizal fungi (ECM) to survive in nature, including whitebark pine. Non-mycorrhizal seedlings are at risk of dying when planted in soil lacking appropriate mycorrhizal fungi; this might include ghost forests, severe burns, dry habitats and areas not previously in pine. This study screened 25 isolates of native ECM fungi (primarily suilloids) from whitebark pine forests in the Greater Yellowstone Area as a preliminary step in development of an effective inoculum for whitebark pine seedlings grown in forest nurseries. Most are 'suilloid' fungi specific for 5-needle pines. A majority grew well *in vitro* and selected strains were then used to develop various types of mycorrhizal inoculum. Four basic inoculation methods were tested under greenhouse conditions using spore slurries and soil inocula. Spore slurries added to soil produced the highest rate of mycorrhizal colonization (100 per cent frequency) in the shortest time (3-5 months), but colonization also occurred with soil inoculum (mycelium). Fertilization was found to suppress mycorrhizal colonization at least at the high levels tested. Soil substrate was found to be an important factor in ECM colonization and some soil mixes suppressed or prevented colonization. Commercial inoculum is not recommended since it risks introduction of alien fungi, it may not favor 5-needle pines, and therefore has the potential to disturb sensitive whitebark pine systems. Here we report results from preliminary trials and discuss on-going research to provide up-to-date information.

## Introduction

Whitebark pine (*Pinus albicaulis* Engelm.) forests are in serious decline due to blister rust, mountain pine beetles, fire suppression and possibly climate change (Schwandt 2006, Smith et al. 2008, Logan and others 2010). In some areas of the western U.S. forests have been reduced 90 percent or more. Restoration efforts have been on-going for over 15 years (Schwandt 2006; Tomback and others 2001) and this includes development of seed germination methods (Burr and others 2001), nursery production of whitebark pine seedlings (Burr and others 2001; Riley and others 2007), selection of rust resistant strains (Mahalovich and Dickerson 2004), research on seedling diseases (Dumroese 2008), development of cone collection techniques, improvement of planting methods (McCaughey and others 2010) and use of burned sites for out-plantings (Keane and Arno 2001; Keane and Parsons 2010). Over 200,000 nursery seedlings have been planted in the western U.S. and survival rates are low in many areas. Izlar (2007) found a 42 percent overall survival rate for the 100,000 seedlings assessed; in some areas survival approached zero. One neglected area of vital research which has the possibility of enhancing seedling survival on out-planting is inoculation of nursery seedlings with mycorrhizal fungi.

All pines, including whitebark pine, require ectomycorrhizal (ECM) fungi to survive in nature (Smith and Read 1997). These fungi enhance pine survival by providing nutritional benefits, imparting drought tolerance and offering protection from pathogens and soil grazers (Cripps 2002, 2004; Molina and Trappe 1984). In nature, non-mycorrhizal seedlings are at risk in soil lacking appropriate mycorrhizal fungi. Therefore mycorrhizal fungi should be considered in nursery or silvicultural methods and in monitoring out-planted seedling performance (Landis and others 1990, Khasa and others 2009). For nursery methods, the USFS handbook (Landis and others 1990) and 'Advances in Mycorrhizal Science and Technology' (Khasa and others 2009) both recommend that mycorrhizal inoculation be tested on a small scale before applying to an entire nursery. While mycorrhizal inoculation is routinely used in reforestation efforts (Khasa and others 2009), inoculation methods vary and need to be developed for *each* tree species. This is primarily because it is necessary to match greenhouse regimes required for a particular tree species with those conducive to mycorrhizal colonization by the appropriate fungi. While commercial inoculum is often used, the most effective inoculum is naturally associated with the tree species being inoculated (Davey and others 1990). Therefore, inoculation of whitebark pine seedlings should be with *native* mycorrhizal fungi adapted to local conditions and those known to be important in whitebark pine seedling survival in nature (Mohatt and others 2008). Use of commercial inoculum runs the risk of introducing alien fungi and those not appropriate for whitebark pine.

Inoculation of seedlings can also benefit the nursery as a 'green technology' that reduces fertilizer and irrigation as well as pesticide use, and protects against some pathogens (Whipps 2004). Colonized root systems are often 'bushier' with more secondary roots that are pre-conditioned to exploit soil resources when planted (Khasa and others 2009).



In: Keane, Robert E.; Tomback, Diana F.; Murray, Michael P.; and Smith, Cyndi M., eds. 2011. **The future of high-elevation, five-needle white pines in Western North America: Proceedings of the High Five Symposium.** 28-30 June 2010; Missoula, MT. Proceedings RMRS-P-63. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 376 p. Online at http://www.fs.fed.us/rm/pubs/rmrs_p063.html

In the field, inoculation can enhance seedling survival with the correct combination of host, fungus, soil/substrate and abiotic conditions; results can be dramatic in areas lacking appropriate fungi in the soil (Parlade 2004; Steinfield and others 2003; Stenström and Ek 1990). Inoculation has been calculated to be cost effective when survival increases at least 5 percent (Parlade 2004).

'Host-specific' native mycorrhizal fungi can also be adapted to particular soil and climatic conditions. At the Federal Forest Nursery in Austria, European stone pines (*Pinus cembra* L.) have been inoculated for over 50 years with native suilloid fungi adapted to high elevation conditions. This has dramatically increased the out-planting success rate in these habitats (Moser 1956, Weisleitner, personal communication 2008). A multi-level approach using a combination of intensive silvicultural methods has increased survival of planted *P. cembra* seedlings from ca. 50 to 90 percent and these methods are still employed today (Weisleitner, personal communication 2008). We were able to visit this nursery for direct transfer of information on inoculation and planting techniques for stone pines.

Over 40 species of ectomycorrhizal fungi have been confirmed with whitebark pine on our sites in the Greater Yellowstone Ecosystem (GYE) which contain some of the last remaining intact forests (Cripps and Mohatt 2005; Cripps and others 2008; Mohatt 2006; Mohatt and others 2008). Many are suilloid fungi host-specific on some level (Bruns and others 2002). Individual species are restricted to pine, 5-needle pine, or stone pine; some appear to be strictly associated with whitebark pine. Amazingly, we have found *Suillus sibiricus* and other suilloids also known to occur with stone pines in Europe and Asia which suggests a long co-evolutionary history (Moser 2004). The suilloids (*Suillus* and *Rhizopogon* species) are also of interest because they are known to be important in the establishment of pine seedlings and have been used successfully in nurseries to this effect (Castellano and others 1985; Parladé and others 2004; Rincon and others 2005; Steinfeld and others 2003).

The challenges of using native fungi include 1) selecting native fungi for the nursery that ultimately enhance survival in the field, 2) determining which soil substrates are conducive to mycorrhizal colonization in the nursery, 3) finding fertilizer regimes that do not interfere with mycorrhizal colonization, and 4) avoiding chemicals for pest control (especially fungicides). There are economic challenges as well, but once mycorrhizal inoculation is integrated into normal nursery operations (hopefully with minor adjustments), studies have shown the economic benefits to nurseries can be positive as studies have shown (Davis and others 2009; Parladé and others 2004).

The main goal of the present research is to develop methods for inoculation of whitebark pine seedlings with native ectomycorrhizal fungi under nursery conditions that ultimately improves survival in the field. We have made significant progress in capturing native fungi from whitebark pine forests in the GYE for this project. Here we report initial screening data on 25 strains of native mycorrhizal fungi collected from whitebark pine forests for their potential as inoculum. We also report results of an early trial (Experiment 1) that tested various inoculation methods for efficacy of mycorrhizal colonization in the nursery. Experiments 2 and 3 examined the effects of fertilizer and various soil substrates on mycorrhizal colonization (Table 1). Assessment for these trials is percent colonization of root systems and not increased seedling size in the nursery. Results are discussed in context with our current research to provide as up-to-date information as possible. Goals outside the scope of this report are determining when inoculation is necessary and if inoculation enhances the survival of whitebark pine seedlings in the field.

## Methods

### *Screening of Native Ectomycorrhizal Fungi*

Ectomycorrhizal fungi were collected from whitebark pine forests in the Greater Yellowstone Ecosystem and ecological parameters recorded. Details of locations are in the MSU database of fungal collections (MONT Herbarium and Mohatt and others 2008). A majority are suilloid fungi, *Suillus* and *Rhizopogon*. *Cortinarius, Hygrophorus, Lactarius*

**Table 1.** Components of various soil media types used in various experimental Trials.

| Soil Media | By Volume | pH | Origin | Used in |
|---|---|---|---|---|
| Soil Mix 1: Sunshine mix #1[a], MSU mix[b] & Vermiculite[c] | 1Sm:1M:1V | 6.5 | Mixed at MSU Plant Growth Center | Experiment 1 |
| Soil Mix 2: Sphagnum Peat Moss, MSU mix & Vermiculite | 1P:1M:1V | 5.0 | Mixed at MSU Plant Growth Center | Experiments 1, 2, 3 |
| Canadian sphagnum Peat Moss & Sawdust | 8P: 2S | 5.2 | Original media from USDA nursery[d] in Styrofoam blocks | Experiments 1 & 2 |
| Canadian sphagnum Peat Moss & Vermiculite | 1P: 1V | 7.0 | Mixed at MSU Plant Growth Center | Experiment 3 |
| Canadian Sphagnum Peat Moss and Bark (not composted) | 1P: 1B | 7.3 | Original media from USDA nursery[d] in Styrofoam blocks | Experiment 3 |

[a] SunGrow, Bellevue, WA.
[b] Loam soil, Canadian Sphagnum peat moss, and washed concrete sand are blended in a 1:1:1 by volume ratio, including AquaGro 2000 G wetting agent at one lb./cubic yd; mix steam pasteurized at 70°C for 60 min.
[c] SunGrow, Bellevue, WA.
[d] USDA Forest Service Nursery in Coeur D'Alene, Idaho.

and *Russula* species were not considered for testing since it is known that these genera do not grow *in vitro* and are primarily associated with mature trees and not seedlings. *Laccaria* and *Hebeloma* species, typically used as fungal inoculum, are rare in whitebark pine forests. Fungi as sporocarps (mushrooms/truffles) were identified using classical taxonomic methods; ectomycorrhizae on roots were identified using molecular techniques. The latter includes: DNA extraction, PCR, sequencing of the Internal Transcribed Spacer region followed by comparison to sequences in Genbank or our own DNA library (Mohatt 2006).

Twenty-five strains of native fungi from whitebark pine forests were screened as inoculum for whitebark pine seedlings. Tissue was removed from sporocarps using sterile technique and plated out on Petri dishes of Modified Melin Norkrans media (Brundrett and others 1996). Ectomycorrhizae on roots were surface sterilized with hydrogen peroxide or 10 percent Clorox solution, rinsed with sterile water and plated out on MMN.

The presence or absence of growth *in vitro* was used as an initial screening measure to identify potentially useful fungi. Strains that showed vigorous growth in culture were used to develop various types of soil and liquid fungal inoculum that was applied to whitebark pine seedlings. In addition, spore slurries produced by grinding fresh sporocarps with distilled water and stored at 5º C until application were also tested on seedlings. Out of 25 strains, ten were tested as spore slurries. Also, two new strains of *Suillus sibiricus* (CLC 2421 and CLC 2440) were used in trials. The fungi were then evaluated for their ability to form mycorrhizae on whitebark pine seedling roots in the greenhouse.

## *Whitebark Pine Seedlings for Experiments 1-3*

Approximately 300 two to four-week-old whitebark pine seedlings were obtained from the USDA Forest Service Nursery in Coeur D'Alene, Idaho (Burr and others 2001). Seedling lots were from various locations and included lots 7425 and 7029, and 'extras'. At the Idaho nursery seedlings were originally grown under standard conditions in a substrate mix of Canadian Sphagnum peat moss and sawdust (8:2 by volume) in Styrofoam® blocks (91cells, 130 $cm^3$). Additional pre-germinated whitebark pine seedlings were planted into Ray Leach cone-tainers™ (3.8 cm x 14 cm, 115 $cm^3$) containing soil mix 1 or soil mix 2 after radicals reached a length of approximately 0.5 cm. At the Plant Growth Center (Montana State University), seedlings were subsequently grown under standard greenhouse conditions (22ºC. day and 18ºC night temperatures, 16 hr photoperiod). Seedling root systems were randomly examined before inoculation and 14/15 were free of nursery mycorrhizae such as E-strain or *Thelephora*. One seedling was minimally colonized by a nursery type of ectomycorrhizal fungus.

Several soil substrates were used in the various experiments reported in this paper. The components of these soil types are described in Table 1. Soil mix 1, soil mix 2 and the peat:sawdust mixture were used in Experiment 1. Soil mix 2 and the peat:sawdust mix were used in Experiment 2. Soil mix 2, the peat:vermiculite mix and the peat:bark mix were used in Experiment 3.

### Experiment 1: Comparison of 4 inoculation methods

Selected native mycorrhizal fungi (16 strains) were used to develop various types of inoculum. Four general methods were used in the initial trial as a starting point towards development of a standard method for inoculation of whitebark pine seedlings with native mycorrhizal fungi in the greenhouse. Confounding factors are inherent in this approach (comparisons of whole methods) but gave information which directed follow-up experiments. Trials were also determined by the availability of materials (seeds, seedlings, fungi). Comparisons were made by assessing the frequency and abundance of colonization on seedlings roots by mycorrhizal fungi. Replication was N = 14 for each treatment.

### METHOD 1: Soil inoculum 1 (agar plugs) & seedlings grown in Styrofoam® blocks

Modified Melin Norkrans liquid medium was added at a ratio of 85-100 ml to 250-300 ml of a substrate mixture containing Canadian Sphagnum peat moss and Vermiculite (volume ratio 1:9). The substrate mix was added to Mason jars and sterilized (45 min. at 121ºC). The soil inoculum was prepared by adding 10 colonized agar plugs (0.5 x 0.5 cm) of actively growing mycorrhizal cultures to the sterile substrate mix. The soil inoculum was incubated for 4 to 6 weeks at 20˚C. Seedlings in Styrofoam® blocks in peat:sawdust (8:2) mixture were used for this method. Approximately 5 g of soil were removed from the top layer of the cells with a scoop. Five grams of mycorrhizal inoculum were placed in the created space adjacent to the root system and re-covered with removed soil. Mycorrhizal fungi were allowed to establish and grow for 6 to 10 months before evaluation of fungal colonization.

### METHOD 2: Soil inoculum 2 (liquid) and seedlings grown in Styrofoam® blocks

Liquid cultures were prepared by transferring 8 agar plugs (0.5 x 0.5 cm) of actively growing mycorrhizal cultures to glass flasks containing 150 ml of sterile MMN media. The cultures were placed onto a rotary shaker and grown for 4 to 6 weeks at 20˚C. Liquid cultures were added at a ratio of 85-100 ml to 250-300 ml of a sterile substrate mixture containing Canadian Sphagnum peat moss and Vermiculite (volume ratio 1:9). The soil inoculum was incubated for 4 to 6 weeks at 20 ˚C. Seedlings in Styrofoam® blocks in peat:sawdust (8:2) mixture were also used for this method. As described above, 5 g of mycorrhizal inoculum were added into the created space adjacent to the root system and re-covered with removed soil. Mycorrhizal fungi were allowed to establish as described earlier.

### METHOD 3: Spore inoculum & seedlings grown in Soil Mix 2 in Ray Leach single cells

Mature fruiting bodies of *Suillus sibiricus*, *Rhizopogon subpurpureus*, *Rhizopogon* cf *evadens*, *Rhizopogon* cf *molligleba*, and *Rhizopogon* cf *olivaceofusca* collected in whitebark pine forests in Montana were carefully cleaned. The

hymenium of each was removed, cut in small pieces, and ground separately for 1 min in a coffee grinder with 10 ml of sterile distilled water. The ground materials were diluted into 100 ml sterile distilled water and stored in glass bottles at 4°C. Seedlings in Ray Leach single cells in soil mix 2 were used to test this method. Approximately 5 ml of one type of spore slurry was added to each seedling with a pipette. Mycorrhizal fungi were allowed to establish as above.

#### METHOD 4: Soil inoculum 1 (agar plugs) & seedlings grown in Soil Mix 1 in Ray Leach single cells.

This method is the same as Method 1 except that seedlings were in soil mix 1 and in Ray Leach single cells.

#### Experiment 2: Comparison of types of spore inoculum (with and without fertilizer).

Spore slurries were most effective in Experiment 1, subsequently this method was used to examine the efficacy of various types of spore treatments on mycorrhizal colonization. Treatments included: full strength slurry, slurry diluted 1:10, dried spores, and frozen spores. Spore slurry of one *Suillus sibiricus* strain (CLC 2440) was selected for the trial. In addition, three levels of fertilization were added as three additional treatments plus a control.

For the spore slurries, fresh fruiting bodies were processed as described above for spore slurries. A hematocytometer was used to determine the number of spores per volume, which was generally around $10^6$spores/ml. Spore slurries were used full strength or diluted 1:10 with distilled water. Spores for the 'frozen treatment' came from pieces of sporocarp frozen at 0°C for several weeks and then subjected to the same treatment. Spores for the 'dried treatment' came from sporocarps dried on a dehydrator and subsequently subjected to the grinder. Spore solutions were stored at 4°C and shaken well before use. Approximately 2 ml of the respective spore solutions were applied just below the soil surface close to the root system of seedlings grown in peat:sawdust in Styrofoam® blocks. Control seedlings were not inoculated. Mycorrhizal fungi were allowed to grow for at least 5 months before the root colonization was evaluated. The fertilizer treatments consisted of the application of 200 ppm of NPK (Scotts® Peters General Purpose 20-20-20) applied to saturation once (treatment 1), twice (treatment 2) or three times (treatment 3) a week. N=7 seedlings were used for each treatment.

#### Experiment 3: Effects of soil substrate type on mycorrhizal colonization

Seedlings were planted in three different soil substrates types in Ray Leach containers: peat:non-composted bark, peat:vermiculite and Soil Mix 2 (described in Table 1). Seedlings were inoculated with *Rhizopogon* CLC 2544 and *Suillus sibiricus* strains CLC 2375, 2421 and 2440, using full-strength spore slurry as described above. Controls were not inoculated. Seedlings were inoculated and mycorrhizal fungi were allowed to establish under greenhouse conditions for 5 months before evaluation. N=7 for each treatment.

#### Evaluation of mycorrhizal colonization

Seedlings were carefully extracted from the Styrofoam® blocks or Ray Leach containers. The roots of each seedling were immersed in distilled water and soil particles were removed by gentle agitation. For the non-destructive sampling technique the intact root system of each seedling was placed in petri plates containing distilled water and examined with a dissecting microscope (Nikon SMZ 1500, Meridian Instrument Company, Inc., Kent, WA). Ectomycorrhizal root tips were recognized by the presence of a mantle, extramatricular hyphae or rhizomorphs for some, and the dichotomous branching typical of pines. The frequency of mycorrhizal colonization was determined by presence/absence of mycorrhizae of the fungal strain. Quantification of mycorrhizal colonization was also assessed as either the number of mycorrhizal root tips per seedling or the estimated percentage of the root system that was colonized (0-100 percent) on each seedling (Brundrett and others 1996). Application of statistical analysis was difficult due to the patchy nature of results. Assessment in all trials was non-destructive in that seedlings were able to be transplanted after assessment. Effects on plant parameters were also measured but are not reported here.

## Results

All sixteen of the strains that were tissue cultured onto Petri "plates" grew *in vitro* on MMN media (Table 2, column 6, M+). Six showed vigorous mycelial growth (M++) and were selected for further testing. These included: *Suillus subalpinus* CLC 2341, *S. cf subvariegatus* CLC 2344, *S. sibiricus* CLC 2345, *Suillus sp* CLC 2199, *Rhizopogon subbadius* CLC 2294 and *Cenococcum geophilum* VT 1009. These six were then tested for their ability to grow in "liquid" MMN media and peat:vermiculite (Table 2, columns 7 and 8). All six were able to grow in both substrates and were applied as a liquid or soil based inoculum to seedlings (Table 2, column 9) and all but *Cenococcum* formed mycorrhizae. An additional eight fungal strains were added as spore slurries (Table 2, column 6, S) directly to seedlings; these were primarily over-ripe suilloid fungi not suitable for tissue culturing. All formed mycorrhizae except *Thaxterogaster*.

While it was not possible to test all methods using all fungal strains, Experiment 1 showed that mycorrhizal colonization of whitebark pine seedlings is possible using Methods 1, 2 and 3 (Table 3). However, colonization did not occur with Method 4 using Soil Mix 1; this soil mix was found to be fungal suppressive due to the Sunshine Mix which concurs with results for other trials using this soil mix (not reported here). Mycorrhizal colonization occurred using either a liquid or agar plug initiated soil inoculum, although colonization was 'patchy' (not consistent within a treatment). The spore method produced the highest colonization rate in the shortest time period for all fungi tested with high frequency ratings. There were fungal effects as well with certain strains of *Suillus* out-performing other groups as soil inoculum. With

**Table 2.** Initial screening of native ectomycorrhizal fungi for potential use as inoculum for whitebark pine seedlings as assessed by growth characteristics on various substrates.

| No. | Mycorrhizal species | Location | Source | Host | Plate[a] | Liquid[b] | Soil[c] | Seedling[d] |
|---|---|---|---|---|---|---|---|---|
| CLC 2035 | *Rhizopogon subpurp.* | New World | sporocarp | *P. albicaulis* | M+ | na | na | na |
| CLC 2036 | *Rhizopogon* sp. | New World | sporocarp | *P. albicaulis* | M+ | na | na | na |
| WO 81.1 | *Tricholoma moseri* | New World | sporocarp | *P. albicaulis* | M - | na | na | na |
| Rhiz 1w | *R.* cf *ochraceorubens* | Waterton Park | sporocarp | *P. contorta* | M+ | na | na | na |
| Hyp 1 | *R.* cf *salebrosus* | Waterton Park | sporocarp | *P. flexilis* | M+ | na | na | na |
| GDP 1 | *Rhizopogon.* sp. 1 | Glacier Park | roots | *P. flexilis* | M+ | na | na | na |
| UB 7 | *Rhizopogon* sp. 2 | Fridley Burn | native soil | *P. albicaulis* | M+ | na | na | na |
| CLC 2199 | *Suillus* sp. (veil) | Yellowstone | sporocarp | *P. albicaulis* | M++ | + | + | + |
| CLC 2294 | *R. subbadius* | Yellowstone | sporocarp | *P. flexilis* | M++ | + | + | + |
| CLC 2341 | *S. subalpinus* | New World | sporocarp | *P. albicaulis* | M++ | + | + | + |
| CLC 2344 | *S. cf subvariegatus* | New World | sporocarp | *P. albicaulis* | M++ | + | + | + |
| CLC 2345[a] | *S. sibiricus (thick)* | Yellowstone | sporocarp | *P. albicaulis* | M++ | + | + | + |
| CLC 2345[b] | *S. sibiricus (thin)* | New World | sporocarp | *P. albicaulis* | M+ | na | na | na |
| CLC 2346 | *S.* cf *brevipes* | Yellowstone | sporocarp | Conifers | M - | na | na | na |
| CLC 2347[c] | *S. subalpinus* | Yellowstone | sporocarp | *P. albicaulis* | M+ | na | na | na |
| VT 1009 | *Cenococcum geophil.* | Eastern US | roots | Conifers | M ++ | + | + | - |
| CLC 2375 | *S. sibiricus* | Beartooths | sporocarp | *P. albicaulis* | S | na | na | + |
| CLC 2377 | *R. subpurpurascens* | Beartooths | sporocarp | *P. albicaulis* | S | na | na | + |
| CLC 2379 | *R.* cf *evadens* R 1 | Yellowstone | sporocarp | *P. albicaulis* | S | na | na | + |
| CLC 2380[a] | *R.* cf *molligleba* R2 | Yellowstone | sporocarp | *P. albicaulis* | S | na | na | + |
| CLC 2380[b] | *R.* sp. (yellow) R3 | Yellowstone | sporocarp | *P. albicaulis* | S | na | na | + |
| CLC 2381[a] | *R. olivaceofuscus* 4,5 | New World | sporocarp | *P. albicaulis* | S | na | na | + |
| CLC 2382 | *Thaxterogaster* sp. | New World | sporocarp | *P. albicaulis* | S | na | na | - |
| NW Hyp 1 | *Hypogeous* 1 | New World | sporocarp | *P. albicaulis* | S | na | na | na |
| NW Hyp 2 | *Hypogeous* 2 | New World | sporocarp | *P. albicaulis* | S | na | na | na |

[a] growth on Petri 'plates' of MMN (M+ = growth, M++ = vigorous growth, M- = poor growth).
[b] growth in 'liquid' MMN media (+ = growth, na = not tested).
[c] growth in peat:vermiculite (1:9 v/v) 'soil' mix (+ = growth, na = not tested).
[d] fungi used to inoculate whitebark pine seedlings.
S = spores from fruiting bodies used for direct inoculation of seedlings.

spores *Rhizopogon* species were also able to colonize seedlings at acceptable rates. Seedlings that were well colonized with mycorrhizal fungi exhibited a darker green color and root systems were often more well-developed (data not shown).

In Experiment 2, the application of fertilizer reduced mycorrhizal colonization to almost negligent levels (Figure 1, A and B) regardless of the type of spore inoculum applied. The lightest application (F1) had the highest frequency of colonization compared to heavier doses, but at all three levels, colonization of the overall root system was less than 7 percent and not acceptable. All types of spore inoculum (including slurries, dried or frozen spores) were effective in mycorrhizal colonization when applied without fertilizer with colonization levels up to 23 percent and frequencies of 70-100 percent. Differences in colonization levels were negligible between the full and 1:10 diluted spore slurry. The dried inoculum lagged behind in percent colonization but not in frequency of seedlings infected.

In Experiment 3, there was little mycorrhizal colonization in the peat:bark substrate for all four of the fungal strains tested (Fig. 2). All four fungi colonized seedling roots in both peat:vermiculite and soil mix 2 covering 7-28 percent of the roots systems in a majority of seedlings with high frequencies of 45-100 percent. There was also variation within strains of *Suillus sibiricus*, and results suggest that particular strains had a preference for soil type in this small trial.

## Discussion

The main goal of this project was to initiate the development of an effective method for inoculation of whitebark pine seedlings with native ectomycorrhizal fungi under nursery conditions. We have made significant progress in capturing and storing native fungi from whitebark pine forests in the GYE for this project (a rather difficult task since fungi rarely fruit) and in screening them for potential as inoculum for whitebark pine seedlings. Mycorrhizal colonization was successful with numerous strains of native ectomycorrhizal fungi using several methods in the greenhouse. However, results were inconsistent within treatments (sometimes ranging from 0 to 100 percent colonization). Methods need to be refined for more consistent and reliable mycorrhizal colonization before moving to a larger scale that can be integrated into nursery protocol. However, a small successful trial using older seedlings is reported later in the discussion along with management recommendations.

### *Fungal strains*

A total of 25 strains of native ectomycorrhizal fungi (Table 2) were tested in this initial trial and additional strains have been tested since; this includes native mycorrhizal fungi now being tested on limber pine (*Pinus flexilis* James). We

**Table 3.** Experiment 1: A comparison of four methods used to inoculate strains of ectomycorrhizal fungi onto whitebark pine seedlings in the nursery. Methods are summarized; for details see Table 1 and method section.

| Method | Isolate Number | Fungus | Colonization frequency(%) | Average colonization(%) | Average No. mycorrhizae | Time (months) |
|---|---|---|---|---|---|---|
| **Method 1: Soil inoculum 1 (agar plugs) & seedlings grown in Styrofoam® blocks (in peat:sawdust)** | | | | | | |
| 1 | CLC 2199 | *Suillus* sp. (veil) | 16.7 | <1 | 0.7 | 9 |
| 1 | CLC 2341 | *Suillus subalpinus* | 25.0 | <1 | 0.3 | 9 |
| 1 | CLC 2344 | *Suillus cf subvariegatus* | 16.7 | 0–25 | 19.7 | 6 |
| 1 | CLC 2345[a] | *Suillus sibiricus* | 0.0 | 0 | 0.0 | 9 |
| 1 | CLC 2345[a] | *Suillus sibiricus* | 16.7 | <1 | 0.2 | 10 |
| 1 | CLC 2345 | *Suillus sibiricus* 3 | 0.0 | 0 | 0.0 | 6 |
| 1 | CLC 2345 | *Suillus sibiricus* 3 | 40.0 | <1 | 1.2 | 9 |
| 1 | CLC 2345[b] | *Suillus sibiricus* | 100.0 | 0–25 | 38.9 | 9 |
| 1 | CLC 2345[b] | *Suillus sibiricus* | 100.0 | 25–50 | 47.0 | 10 |
| 1 | CLC 2294 | *Rhizopogon subbadius* | 33.3 | 0–25 | 22.3 | 6 |
| 1 | CLC 2294 | *Rhizopogon subbadius* | 16.7 | <1 | 6.5 | 9 |
| 1 | CLC 2294 | *Rhizopogon subbadius* | 16.7 | <1 | 0.3 | 10 |
| 1 | CLC 2294 | *Rhizopogon subbadius* | 33.3 | 0–25 | 7.2 | 10 |
| 1 | VT 1009 | *Cenococcum geophilum* | 16.7 | <1 | 0.8 | 9 |
| 1 | Control | Control | 0.0 | 0 | 0.0 | 9 |
| **Method 2: Soil inoculum 2 (liquid) & seedlings grown in Styrofoam® blocks (in peat:sawdust)** | | | | | | |
| 2 | CLC 2035 | *Rhizopogon subpurpurascens* | 16.7 | <1 | 4.0 | 9 |
| 2 | CLC 2199 | *Suillus* sp. (veil) | 100.0 | 25-50 | 47.5 | 9 |
| 2 | CLC 2341 | *Suillus subalpinus* | 60.0 | 0-25 | 37.8 | 9 |
| 2 | CLC 2344 | *Suillus cf subvariegatus* | 25.0 | 0-25 | 48.0 | 9 |
| 2 | CLC 2345 | *Suillus sibiricus* 3 | 0.0 | 0 | 0.0 | 9 |
| 2 | CLC 2294 | *Rhizopogon subbadius* | 0.0 | 0 | 0.0 | 9 |
| 2 | CLC 2035 | *Rhizopogon subpurpurascens* | 16.7 | <1 | 4.0 | 9 |
| **Method 3: Spore inoculum & seedlings grown in Soil Mix 2 in Ray Leach single cell containers** | | | | | | |
| 3 | CLC 2375 | *Suillus sibiricus* | 100.0 | 25-50 | 49.0 | 5 |
| 3 | CLC 2377 | *Rhizopogon subpurpascans* | 100.0 | 25-50 | 30.0 | 5 |
| 3 | CLC 2379 | *Rhizopogon* cf *evadens* | 100.0 | 0-25 | 6.0 | 5 |
| 3 | CLC 2380[a] | *Rhizopogon* cf *molligleba* | 100.0 | 25-50 | 33.7 | 5 |
| 3 | CLC 2381 | *Rhizopogon* cf *olivaceofusca* | 100.0 | 25-50 | 59.3 | 5 |
| **Method 4: Soil inoculum 1 (agar plugs) & seedlings in Soil Mix 1, Ray Leach single cell containers** | | | | | | |
| 4 | CLC 2035 | *Rhizopogon subpurpurascens* | 0.0 | 0 | 0.0 | 9 |
| 4 | CLC 2199 | *Suillus* sp. (veil) | 0.0 | 0 | 0.0 | 9 |
| 4 | CLC 2341 | *Suillus subalpinus* | 0.0 | 0 | 0.0 | 9 |
| 4 | CLC 2344 | *Suillus cf subvariegatus* | 0.0 | 0 | 0.0 | 9 |
| 4 | CLC 2345 | *Suillus sibiricus* 3 | 16.7 | <1 | 0.5 | 9 |
| 4 | CLC 2294 | *Rhizopogon subbadius* | 0.0 | 0 | 0.0 | 9 |
| 4 | VT 1009 | *Cenococcum geophilum* | 0.0 | 0 | 0.0 | 9 |

have found suilloids in all whitebark pine studied (Mohatt and others 2008) and as a dominant group on seedlings roots (Mohatt 2006). Also, whitebark pine seedlings planted for various management strategies such as after fire (Trusty and Cripps 2010) and along Dunraven Pass in Yellowstone National Park (Cripps and Trusty 2007) also hosted suilloid fungi. This suggests that suilloid fungi specific to 5-needle pines are important in whitebark pine systems and are multi-stage fungi appropriate for young seedlings as well as mature trees.

Results from additional fungal strains suggest a large variety of suilloids can be used as inoculum as long as they occur with 5-needle pines. While some strains of *Suillus* and *Rhizopogon* out-performed other strains in the trials reported here (particularly *Suillus sibiricus*), we have subsequently found that strain performance is also dependent on inoculum type, soil substrate, pH of the system, fertilizer regime, and other conditions. The inconsistent results where some seedlings were 100 percent colonized with no colonization for others within a treatment suggest seedling genetics may also play a role. Caution is therefore advised in limiting selection to just a few strains. Also, we do not yet know if the strains that perform well in the nursery enhance survival on out-planting, however field trials are underway. As stated before, often the most effective inoculum comes from beneath the tree species being inoculated (Davey and others 1990). We are recommending regional sources of fungal inoculum be identified and restricted to particular growing regions for whitebark pine management.

A primary fungus in commercial inoculum, *Rhizopogon roseolus,* associates more with lodgepole pine, while the main species in whitebark pine systems in the Greater Yellowstone Area are ecotypes of *R. evadens* and *R. milleri* (Mohatt and others 2008). Inoculum with *Paxillus involutus* or *Scleroderma*

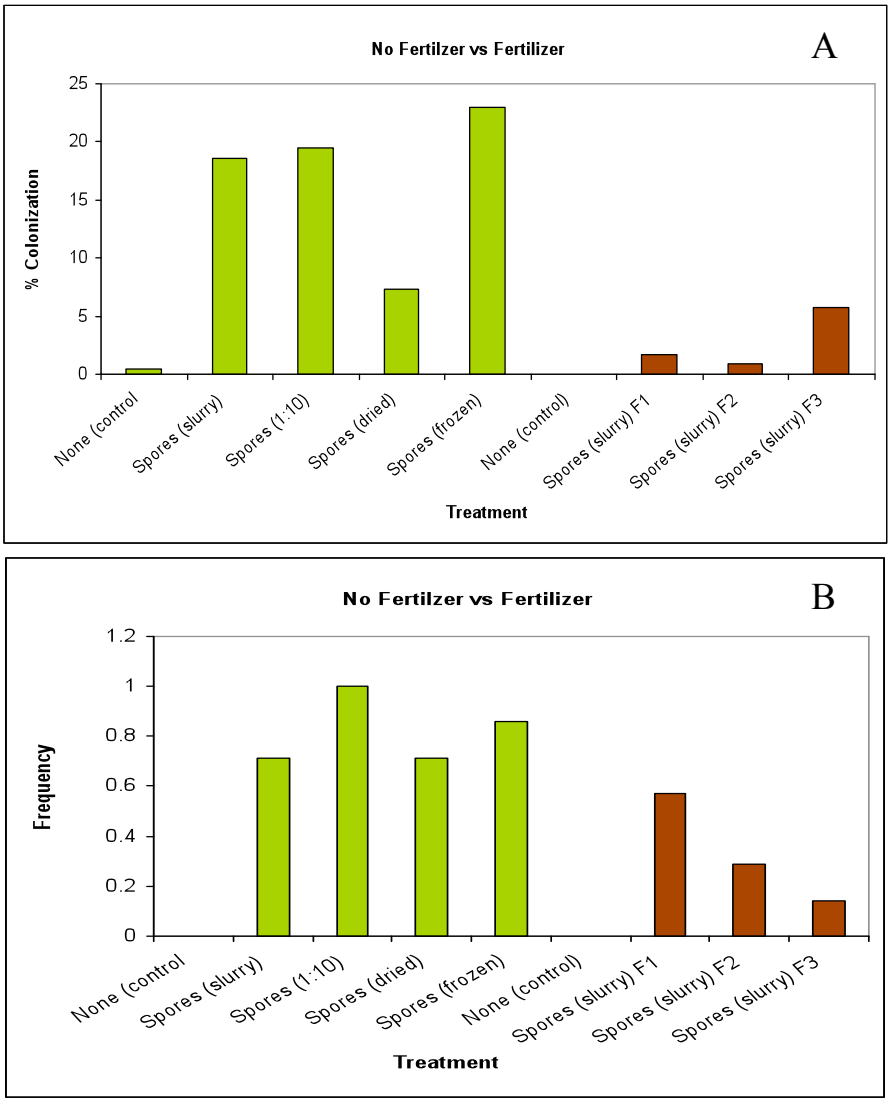


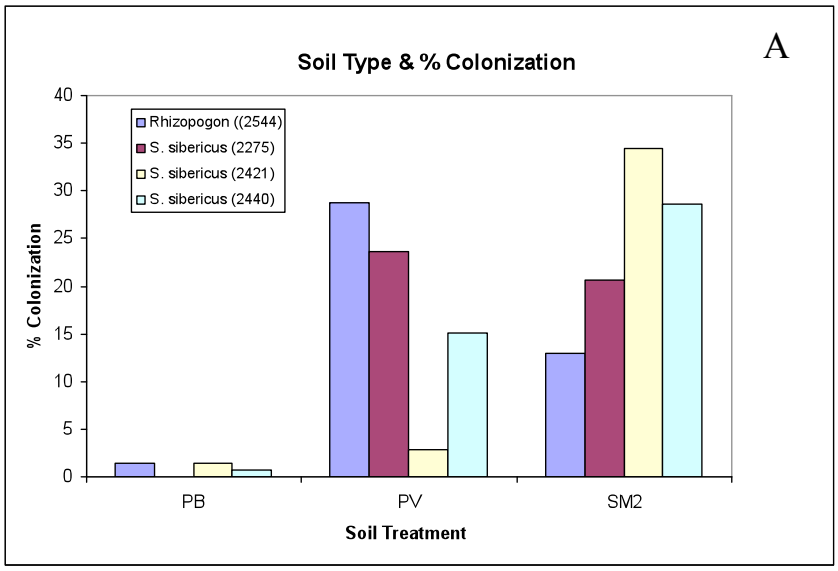

**Fig. 1.** Experiment 2, mycorrhizal colonization of whitebark pine seedlings after inoculation with various types of spore slurry with and without fertilizer (F1=once a week, F2=twice a week, F3=three times a week). A. % colonization of root system. B. frequency of colonization (no. seedlings colonized).

species is not recommended as these fungi are for acidic soils and are not known in whitebark pine systems. Similarly, *Hebeloma* species, often used in commercial inoculum, have only been recorded once on our whitebark pine sites. Alien fungi risk alteration of the food chain since small and large mammals depend on particular suilloids for food in these sensitive systems (Ashkannejhad and Horton 2005; Izzo and others 2005). In addition, the specific physiology of the native fungi may not be functionally redundant with that of those in commercial inoculum. Commercial inoculum could also serve to promote other tree species.

## *Inoculation Methods*

Mycorrhizal colonization of whitebark pine was successful using either spore slurries or soil (mycelial) inoculum; two of the three soil inoculation methods tested showed the potential to be used with whitebark pine seedlings. Fresh spore slurries (method 3) were the most effective method tested resulting in 100 percent colonization of all seedlings inoculated with suilloids. This method is simple and spores can easily be directly added to seedlings in Styrofoam® blocks or Ray Leach containers. A drawback is that fresh spore slurries are not always available at inoculation time. These fungi fruit and produce spores in the fall and seedlings were inoculated directly afterwards resulting in high colonization rates. However, fruiting does not occur every year and it is often difficult to get to these locations at the correct time (Mohatt 2006). These high elevations sites are prone to drought which prevents fungal fruiting. Inoculation in spring would allow colonization just before fall planting. Therefore, we tested reduced amounts of spores (dilution of slurries) and storage methods for spores (freezing and drying). All of these treatments resulted in mycorrhizal colonization and methods need to be refined with larger trials. In subsequent trials, we learned that some spore slurries maintain viability for several months, however colonization was not always as consistent as in the preliminary trial if older or dried inoculum is used. We are currently testing the shelf life of spore slurries.

The soil inoculum also produced mycorrhizae in the greenhouse whether initiated with agar plugs or liquid medium. Mixing soil inoculum into the substrate when possible would likely improve colonization, but this may not be



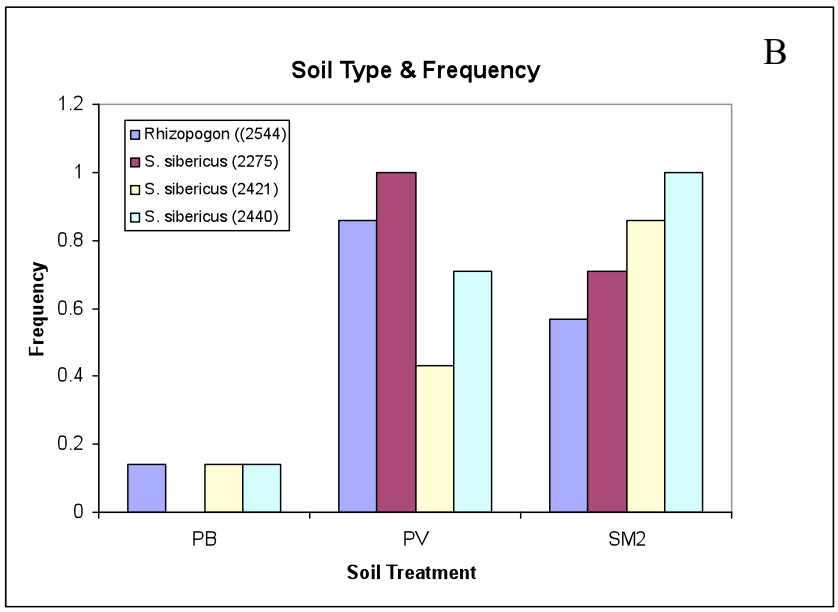




**Fig. 2.** Experiment 3, mycorrhizal colonization of whitebark pine seedlings after inoculation with 4 strains of fungi in three soil substrate types: peat:non-composted bark (1:1), peat:vermiculite (1:1) and Soil Mix 2 (p:MSU mix:v, 1:1:1). A. % colonization of root system. B. frequency of colonization (no. seedlings colonized).

feasible under most nursery situations. Liquid inoculum has drawbacks including the tendency for contamination. The benefit of using a soil inoculum is that it contains only the fungus of interest, it is pathogen free, and may be generated in the nursery. Unlike spore inoculum, genetic diversity of the fungus is kept to a minimum. Also, fungal sporocarps do not need to be collected each year. In a survey of many large scale trials, Brundrett and others (2005) found that regardless of conditions and fungi, mycelial slurries produced 35 percent colonization of root systems of Eucalyptus seedlings and spore suspensions 49 percent, with the latter being more cost effective when applied on a large scale in the nursery. Our data suggest this proportion may apply to whitebark pine systems as well.

## *Substrate Effects*

There is a concern that certain types of substrate may not be amenable to mycorrhizal colonization. In Experiment 1, method 4, Soil Mix 1 (containing Sunshine Mix) prevented mycorrhizal colonization possibly due to the high pH level. Therefore, we tested soil substrates to determine which are suppressive and which conducive to mycorrhizal colonization. In Experiment 3, we tested three soil substrates with four strains of native mycorrhizal fungi to examine their effect on mycorrhizal colonization. These trials were done without fertilizer. The peat:bark precluded mycorrhizal colonization of native fungi and promoted pathogenic and nursery ECM fungi such as *Thelephora* and will not be used in future trials. Both the peat:vermiculite substrate and

**Fig. 3.** Successful mycorrhizal colonization of whitebark pine seedlings with a native suilloid fungus. The seedling was inoculuated at the MSU Plant Growth Center under nursery conditions. White areas on branched short roots are ectomycorrhizae of the fungus. Inset shows the fungus covering short roots and mycelium extending into the soil.

Soil Mix 2 were conducive to mycorrhizal colonization by suilloid fungi for the four strains tested. Experiments since have suggested that a different soil mix 3 (1:1:1 by volume, MSU mix: vermiculite: peat) and a peat:composted bark mix recently used at the Idaho nursery may be preferable for mycorrhizal colonization. The latter has been found to be acceptable for growth of whitebark pine seedlings (Eggleston, personal communication). One factor may be that substrate pH is around 5.5 which is recommended for conifers and also for many ectomycorrhizal fungi.

Possible soil substrates are currently being tested at the Idaho nursery for whitebark pine both to save money (primarily on peat) and to promote growth. Davis and others (2009) report that the peat:bark mixture was preferable to the peat:sawdust mixture for larch. Both peat:sawdust and peat:composted bark have also been tested for whitebark pine (Kent Eggleston, personal communication) and while colonization can occur in either substrate, new evidence from our lab suggests the latter may be preferable for inoculation purposes.

Parladé and others (2004) found that pines inoculated with *Rhizopogon* could be colonized in peat:bark (1:1 by volume) and peat:vermiculite (1:1 by volume), but that pines in the bark mixture benefited more from inoculation which increased survival by 23 percent in the field. However, Rincon and others (2005) found that *Pinus taeda* seedlings in a 1:1 peat:composted bark mix had reduced colonization of *Rhizopogon,* while those in peat:vermiculite were 80 percent colonized. This again suggests that methods need to be developed for each tree species and system.

## *Fertilizer Effects*

In Experiment 2, the fertilizer added at 200 ppm of 20:20:20 once/twice/three times a week was detrimental to the seedlings (browning needle tips) and promoted infection of *Thelephora* which is a greenhouse strain of mycorrhizal fungus that can cause 'choke disease'. We initially wanted to examine high levels of fertilization to check the 'cap' on fertilization, but found that even at fertilization level 1 (once a week), mycorrhization was highly suppressed. In subsequent experiments, we found that some suilloid fungi can tolerate a light fertilizer treatment.

While fertilization is known to reduce mycorrhization, it is possible under some fertilization regimes (Khasa and others 2001). Reducing fertilization to once every 15 days can allow both mycorrhization and good seedling growth (Khasa and others 2001). Also, application of higher levels of inoculum can overcome suppression by liquid fertilizer but not that caused by the time-release fertilizer Osmocote (Castellano and others 1985). It may be that constant release of nutrients prevents spore germination or changes pH. Also, different fungal strains vary in their tolerance to fertilizer (level and type) and need to be tested individually. Davey and others (1990) suggest that ectomycorrhizae can form with some fertilization but might overload seedlings with phosphate and depress growth. The use of exponential fertilization has been shown to not only save on fertilizer use (45 percent less!) for *Pinus monticola* (Dumroese and others 2005) but to also be conducive to mycorrhizal colonization (Quoreshi and Timmer 1998); this offers a possible method for whitebark pine inoculation.

In a recent experiment we inoculated (spore slurries) 16 month old whitebark pine seedlings that had been grown in typical conditions at the Coeur D'Alene Nursery. Fertilization was stopped one month before inoculation to help promote colonization. Stunningly, a majority of seedlings were found to be well colonized after only two months and ectomycorrhizae covered a majority of their root systems (Fig. 3)! If this method can be shown to give consistent results (effective colonization) it would be a 'simple' way to inoculate whitebark pine seedlings. Large adjustments to typical nursery regime would not be necessary. The new peat:composted sawdust media used by the Coeur D'Alene was shown to be conducive to mycorrhizal colonization in this small trial.

## *Survival in the Field*

The older seedlings described above are now out-planted in Waterton-Glacier International Peace Park and survival will be assessed in the next two years. The ultimate goal is to increase survival in the field. This has been shown to be possible under certain circumstances, primarily where natural inoculum is lacking (Wiensczyk and others 2002). However, inoculation can 'jump-start seedlings where appropriate

fungi are found in the soil and when there is replacement by other fungi (Davey and others 1990).

For ponderosa pine seedlings inoculated with *Rhizopogon* survival increased on a dry, harsh site from 71 percent to 93 percent, a 22 percent increase, but inoculation did not make a difference on a second site (Steinfeld and others 2003). Parladè and others (2004) report a 23 percent increase in survival for *Pinus taeda* inoculated with *Rhizopogon* after 43 mo. and found inoculation to be cost effective. Seedling size is sometimes a concern at out-planting. Stenström and Ek (1990) found that while colonized pine seedlings were smaller than controls at planting, they were 50 percent larger after 2.5 years. Inoculation often produces 'bushier' root systems that may be pre-conditioned to soil exploration on out-planting (Khasla and others 2009). This would be in contrast to the root systems we have examined in out-plantings of whitebark pine that have retained their container shape for at least 5 years (Trusty and Cripps 2010).

For whitebark pine, the use of suilloid fungi specific to 5-needle pine could possibly give these pines a competitive edge over other pine species and fir. Therefore, it is recommended that regionally-appropriate native mycorrhizal fungi be used for inoculation of nursery grown whitebark pine seedlings. Preservation of native strains is also important as a management tool as ecotypes are likely to disappear in areas where forests decline. Determining when inoculation is deemed necessary is outside the scope of the present report but see Wiensczyk and others (2002).

## Current Recommendations

Currently we are recommending that managers minimize practices detrimental to soil microbes, seedlings be planted within a year of disturbances before ECM viability declines, seedlings be planted near inoculum sources (living whitebark pines or in soil previously in whitebark pine) and planted seedlings be monitored for mycorrhizal colonization. In areas where native ECM fungi specific for whitebark pine are likely to be absent, inoculation of seedlings in the greenhouse should be considered. These areas include severe burns, areas not previously in whitebark pine, ghost forests, and areas where planted whitebark pine seedlings have a low survival rate. We recommend that only regionally-appropriate native mycorrhizal fungi be used for inoculation of nursery grown whitebark pine seedlings. Commercial mycorrhizal inoculum *should not* be used in sensitive whitebark pine systems to minimize the risk of importing alien fungi. At present our data suggest that older seedlings can be inoculated 3-4 months before out-planting if fertilization is reduced one month before inoculation if the soil media is appropriate.

## Acknowledgments

This research is funded by a U.S. Department of Agriculture, Forest Service Forest Health and Protection grant through the Whitebark Pine Restoration Projects Program (2008-2010), in particular we thank John Schwandt and Kent Eggleston for their help. Additional funding is now provided by Parks Canada through Cyndi Smith at the Waterton Lakes-Glacier International Peace Park for part of this project and for inoculation of limber pine.

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