Inoculation and successful colonization of whitebark pine seedlings with *native* mycorrhizal fungi under greenhouse conditions

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Abstract

Whitebark pine (*Pinus albicaulis* Engelm.) forests are in serious decline due to blister rust, mountain pine beetles, fire suppression and possibly climate change. These pines form forests at tree-line and are important in watershed dynamics, as early colonizers and keystone species that provide habitat, and as a critical food source (pine nuts) for grizzly bears. Restoration efforts to save or regenerate whitebark pine forests 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 in many areas. One possibility for enhancing seedling survival is the application of beneficial mycorrhizal fungi in the greenhouse before out-planting. This study screened 26 isolates of native mycorrhizal fungi from whitebark pine forests in the Greater Yellowstone Area for use as inoculum. A majority grew well *in vitro* and those exhibiting vigorous growth were used to inoculate seedlings. Four methods were tested in the greenhouse. Spore slurries produced the highest rate of mycorrhizal colonization in the shortest time (5 months), soil inoculum produced lower rates of colonization in 9 months, and there was little colonization in particular soil mixes. There was a strong fungal effect and particular strains of *Suillus* and *Rhizopogon* were prolific colonizers under particular conditions; all are specific for 5-needle pines. These strains improved root development and needles were dark green in contrast to controls. A light fertilizer application did not have negative effects on colonization. The pros and cons of using spore slurries versus soil inoculum are discussed. These trials are an initial phase to be followed by development of a 'reliable' method for more consistent colonization for larger scale inoculation of whitebark pine seedlings before out-planting. This would be necessary in areas that lack appropriate native mycorrhizal fungi such as ghost forests, severe burns, and areas not previously in pine. Commercial inoculum should not be used as they have the potential to upset sensitive whitebark pine systems and most do not favor 5-needle pines.

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). In some areas of the western U.S. forests have declined 90% or more. Restoration efforts have been ongoing for over 15 years (Tomback et al. 2001) and include development of seed germination methods (Burr et al. 2001), nursery production of whitebark pine seedlings (Burr et al. 2001), selection of rust resistant strains (Mahalovich and Dickerson 2004), research on seedling diseases (Dumroese 2008), and use of burned sites for out-plantings (Keane and Arno 2001). Over 200,000 nursery seedlings have been planted in the western U.S. and survival rates are low in many areas (Izlar 2007). One neglected area of research is the application of mycorrhizal fungi to nursery seedlings before out-planting to enhance seedling survival.

All pines, including whitebark pine, need ectomycorrhizal fungi to survive in nature (Smith & Read 1997). These fungi enhance survival by providing nutritional benefits, imparting drought tolerance and offering protection from pathogens & soil grazers (Cripps 2002, 2004). In nature,

non-mycorrhizal seedlings are at risk when planted in soil lacking appropriate mycorrhizal fungi. Therefore the presence of appropriate mycorrhizal fungi must be a major consideration for evaluating seedling performance (monitoring) and in silviculture methods for mycorrhizal inoculation of nursery pines (Landis et al. 1990). The USFS handbook recommends that mycorrhizal techniques be tested on a small scale before trying to inoculate an entire nursery. Greenhouse methods for fungal inoculation vary in success and need to be developed for *each* tree species (Landis et al. 1990). Methods for inoculation of whitebark pine should include the use of native mycorrhizal fungi important to whitebark pine seedling survival in nature (Mohatt et al. 2008).

Ectomycorrhizal fungi as sporocarps are difficult to find in whitebark pine forests at tree-line. However, 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 & Mohatt 2005, Mohatt 2006, Cripps et al. 2008, Mohatt et al. 2008). Many of these are suilloid fungi that are host-specific on some level (Bruns et al. 2002). Individual species are restricted to pine, 5-needle pine, or stone pine. The suilloids (*Suillus, Rhizopogon*) are also of interest because this group is known to be important in the establishment of pine seedlings and they have been successfully used in nurseries to this effect (Steinfeld et al. 2003). In Austria, stone pines have been inoculated for over 50 years with native suilloid fungi which has dramatically increased the out-planting success rate at high elevations (Moser 1956, Weisleitner, pers. comm. 2008). Commercial inocula should not be used in sensitive whitebark pine systems and it is therefore important to capture native fungi that can be used in the nursery when inoculation is deemed necessary. This would include ghost forests, mixed conifers with minimal whitebark pine, areas not previously in whitebark pine, severe burns, and areas that lack an accessible source of mycorrhizal inoculum.

The main goal of this project is to develop methods for inoculation of whitebark pine seedlings with native ectomycorrhizal fungi under nursery conditions. We have made significant progress in capturing native fungi from whitebark pine forests in the GYE for this project. Objectives are to 1) evaluate native fungi collected from whitebark pine forests for their potential as inoculum and 2) compare inoculation methods for efficacy of mycorrhizal colonization.

Methods

OVERVIEW

Twenty-six strains of native fungi from whitebark pine forests were initially screened for use as inoculum for whitebark pine seedling using vigorous growth *in vitro* as a primary criterion. Selected native mycorrhizal fungi were then used to inoculate whitebark pine seedlings using four methods that include the use of soil inoculum and spore slurries in various substrates. Three native suilloid fungi were selected for more in depth trials to examine the affect of light fertilization on mycorrhizal colonization. Seedlings were maintained in the Plant Growth Center at Montana State University for several months under standard conditions. Roots were assessed for mycorrhizal colonization and the effects of inoculation were measured for plant parameters. Results were analyzed to determine the most effective treatments.

CAPTURE OF NATIVE ECTOMYCORRHIZAL FUNGI

Ectomycorrhizal fungi were collected from whitebark pine forests in the GYE and ecological parameters were recorded. Details of locations are in the MSU database of fungal collections (MONT Herbarium). Fungi as sporocarps (mushrooms/truffles) were identified using classical taxonomic methods and as ectomycorrhizae on roots using molecular techniques (DNA extraction, PCR, sequencing ITS region and BLAST search or comparison to our own DNA

library) (Mohatt et al. 2006). Tissue was removed from sporocarps using sterile technique and plated out on Petri dishes of Modified Melin Norkrans media (Brundrett et al. 1996). Ectomycorrhizae were surface sterilized with hydrogen peroxide or 10% Clorox solution and plated out on MMN (Fig 1). The presence or absence of growth *in vitro* was used as an initial screening for fungi potentially useful in inoculum development. Fungi which showed vigorous growth in culture were selected for further testing. In all 26 isolates were tested, 10 as spore slurries.

WHITEBARK PINE SEEDLINGS

Approximately 300+ two to four-week-old whitebark pine seedlings were obtained from the USDA Forest Service Nursery in Coeur D'Alene, Idaho (Burr et al. 2001). Seedling lots were from various locations and included lots 7425 and 7029, and 'extras'. Seedlings were originally grown under standard nursery conditions in a substrate mix of Canadian Sphagnum peat moss and sawdust (80:20) in Styrofoam® blocks (91cells, 130 cm^3). In addition 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 the radicals reached a length of approximately 0.5 cm. The seedlings were grown under standard greenhouse conditions at the Plant Growth Center (PGC) at Montana State University. Seedlings were randomly examined for nursery mycorrhizae before being inoculated with mycorrhizal fungi.

PLANTING SUBSTRATES

Three planting substrates were tested:

Canadian Sphagnum peat moss and sawdust (80:20), pH 5.2 in the original Styrofoam® blocks from the USDA Forest Service Nursery in Coeur D'Alene, Idaho (Eggleston, pers. comm.).

Soil mix 1 consisted of Sunshine Mix # 1 (SunGrow, Bellevue, WA), MSU mix (Mineral soil, Canadian Sphagnum peat moss, and washed concrete sand are blended in a 1:1:1 by volume ratio) and Vermiculite (SunGrow, Bellevue, WA) in a volume ratio of 1:1:1. The pH of soil mix 1 was adjusted to 6.5.

Soil mix 2 consisted of Canadian Sphagnum peat moss, MSU mix, and Vermiculate in a volume ratio of 1:1:1 with a pH of 5.0.

INOCULUM TYPES

Soil inoculum 1: Modified Melin Norkrans liquid medium was added at 85 to 100 ml to 250 to 300 ml of a substrate mixture containing Canadian Sphagnum peat moss and Vermiculate (volume ratio 1:9). The substrate mix was filled into Mason jars and sterilized. The soil inoculum was prepared by adding 10 colonized agar plugs 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.

Soil inoculum 2: 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 of sterile modified Melin Norkrans (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 85 to 100 ml to 250 to 300 ml of a sterile substrate mixture containing Canadian Sphagnum peat moss and Vermiculate (volume ratio 1:9). The soil inoculum was incubated for 4 to 6 weeks at 20°C.

Spore inoculum: Mature fruiting bodies of *Suillus sibiricus*, *Rhizopogon subpurpureus*, *Rhizopogon* cf *evadens*, *Rhizopogon* cf *molligleba*, and *Rhizopogon* cf *olivaceofusca* were collected in whitebark pine forests in Montana. The fruiting bodies were carefully cleaned, cut in small pieces, and separately ground 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.

MYCORRHIZAL INOCULATION METHODS

Soil inoculum: approximately 5 g of soil were removed from the top layer of the cells or containers. Five grams of soil inoculum were added into 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.

Spore inoculum: The spore solutions were shaken well before use. Approximately 2 ml of the respective spore solutions were applied 1 inch below the soil surface close to the root system to seedlings grown in Ray Leach containers. Mycorrhizal fungi were allowed to grow for 5 months before the root colonization was evaluated.

METHODS FOR SEEDLING EXPERIMENTS

Four methods were used in initial trials as a starting point towards development a standard method for inoculation of whitebark pine seedlings with mycorrhizal fungi. Each method was dependent on fungi and substrates available for each trial at time of inoculation. Confounding factors are inherent in this approach for comparisons of whole methods but give direction for follow-up experiments. Statistical analysis was possible for variables within each method.

METHOD 1: Soil inoculum 1 (agar plugs) & seedlings grown in Styrofoam® blocks
METHOD 2: Soil inoculum 2 (liquid) and seedlings grown in Styrofoam ® blocks
METHOD 3: Spore inoculum & seedlings grown in soil mix 2 in Ray Leach single cells
METHOD 4: Soil inoculum 1 (agar plugs) & seedlings grown in soil mix 1 in Ray Leach single.

MYCORRHIZAL EVALUATION

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, extramaticular hyphae or rhizomorphs for some, and the dichotomous branching typical of pines. Root tips of each mycorrhizal fungus were counted to determine frequency and quantity (number of tips, % of root system) of mycorrhizal colonization within each sample (Brundrett et al. 1996).

ASSESSEMENT OF PLANT EFFECTS

Development of shoots and root systems were evaluated at the time of mycorrhizal evaluation. A rating scale was used for root and lateral root development with 1= poor development, 2= moderate developed, 3= well developed. The rating scale for the shoot development was 1= weak shoots, 2= moderate shoot development, 3= robust shoot development. The color of the pine needles was also evaluated using the following scale: 1 = dead, 2 = 100 % necrosis, 3 = partial necrosis on lower needles, 4 = partial chlorosis, and 5 = green needles. Non-destructive techniques and the slow growth exhibited by whitebark pine seedlings precluded comparisons of additional parameters. A significant growth response was not expected. Seedlings were then transplanted after assessment for further trials in the greenhouse.

STATISTICAL ANALYSIS

The greenhouse assays were arranged with 6 replications per treatment. Statistical analysis was conducted by analysis of variance (ANOVA) using the general linear model procedure (GLM) of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary NC). The treatment means were separated using Fisher's protected least significant difference test at P =

0.05. Variables tested included a light fertilizer treatment (NPK 20-20-20 mixed at 25 pmm) given three times per week, and controls with/without fertilizer and with/without inoculation. Colonization and plant response was analyzed statistically.

Results

A total of 26 strains of native ectomycorrhizal fungi were collected for initial screening primarily from whitebark pine forests in the Greater Yellowstone Ecosystem and most were suilloid fungi (Table 1). *Cortinarius, Hygrophorus, Lactarius* 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, have not yet been confirmed with whitebark pine. All sixteen of the strains tissue-cultured onto Petri "plates" grew *in vitro* on modified MMN media (Table 1, column 6, M). Six showed vigorous growth and were selected for further testing with seedlings i.e. CLC 2241 *Suillus subalpinus*, CLC 2344 *S. variegatus*, CLC 2345 *S. sibiricus*, CLC 2199 *Suillus sp*., CLC 2294 *Rhizopogon subbadius*, and VT *Cenococcum geophilum.* These six were then tested for their ability to grow in "liquid" MMN culture and peat:vermiculite "soil" (Table 1, columns 7 & 8). All six were able to grow in both of these substrates and were used as liquid or soil inoculum to inoculate seedlings (Table 1, column 9). An additional eight fungi were ground into spore slurries (Table 1, column 6-S) and added directly to seedlings; these were primarily over-ripe suilloid fungi not suitable for tissue culturing. All spore slurries were then added to the seedlings maintained in the greenhouse. Slurries were also incubated to test shelf life (not reported).

Table 1. 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+	-	-	-
CLC 2036	*Rhizopogon* sp.	New World	sporocarp	*P. albicaulis*	M+	-	-	-
WO 81.1	*Tricholoma moseri*	New World	sporocarp	*P. albicaulis*	M -	-	-	-
Rhiz 1w	*R.* cf *ochraceorubens*	Waterton Park	sporocarp	*P. contorta*	M+	-	-	-
Hyp 1	*R.* cf *salebrosus*	Waterton Park	sporocarp	*P. flexilis*	M+	-	-	-
GDP 1	*Rhizopogon.* sp. 1	Glacier Park	roots	*P. flexilis*	M+	-	-	-
UB 7	*Rhizopogon* sp. 2	Fridley Burn	native soil	*P. albicaulis*	M+		-	-
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. variegatus*	New World	sporocarp	*P. albicaulis*	M++	+	+	+
CLC 2345a	*S. sibiricus (thick)*	Yellowstone	sporocarp	*P. albicaulis*	M++	+	+	+
CLC 2345b	*S. sibiricus (thin)*	New World	sporocarp	*P. albicaulis*	M+	-	-	-
CLC 2346	*S.* cf *brevipes*	Yellowstone	sporocarp	Conifers	M -	-	-	-
CLC 2347c	*S. subalpinus*	Yellowstone	sporocarp	*P. albicaulis*	M+	-	-	-
VT 1009	*Cenococcum geophil.*	Eastern US	roots	Conifers	M ++	+	+	+
CLC 2375	*S. sibiricus*	Beartooths	sporocarp	*P. albicaulis*	S	N/A	N/A	+
CLC 2377	*R. subpurpurascens*	Beartooths	sporocarp	*P. albicaulis*	S	N/A	N/A	+
CLC 2379	*R.* cf *evadens* R 1	Yellowstone	sporocarp	*P. albicaulis*	S	N/A	N/A	+
CLC 2380a	*R.* cf *molligleba* R2	Yellowstone	sporocarp	*P. albicaulis*	S	N/A	N/A	+
CLC 2380b	*R.* sp. (yellow) R3	Yellowstone	sporocarps	*P. albicaulis*	S	N/A	N/A	+
CLC 2381a	*R. olivaceofuscus* 4,5	New World	sporocarp	*P. albicaulis*	S	N/A	N/A	+
CLC 2382	*Thaxterogaster* sp.	New World	sporocarp	*P. albicaulis*	S	N/A	N/A	+
NW Hyp 1	*Hypogeous* 1	New World	sporocarp	*P. albicaulis*	S?	N/A	N/A	-
NW Hyp 2	*Hypogeous* 2	New World	sporocarp	*P. albicaulis*	S?	N/A	N/A	-
XX07	*Rhizopogon* sp.	Yellowstone	grizzly scat	*P. albicaulis*	S	N/A	N/A	+

[a] growth on Petri 'plates' of MMN (M+ = growth, M++ = vigorous growth, M- = poor growth).

[b] growth in 'liquid' MMN media (+ = growth, - = no growth).

[c] growth in peat:vermiculite (1:9 v/v) 'soil' mix (+ = growth, - = no growth).

[d] fungi used to inoculate whitebark pine seedlings.

S = spores from fruiting bodies used for direct inoculation of seedlings.

While it was not possible to test all methods for all fungal strains, these initial trials show that mycorrhizal colonization of whitebark pine seedlings was possible using Methods 1-3 (Table 2). Thus it is possible to use pure cultures in agar plugs or liquid agar to produce a soil inoculum that results in viable mycorrhizae, although colonization was 'patchy' with methods 1 & 2.

Table 2. Comparison of inoculation methods on mycorrhizal colonization for different strains of fungi.

Method 1: Soil inoculum 1 (agar plugs) & seedlings grown in Styrofoam® blocks (in peat:sawdust).

Isolate Number	Fungus	Colonization frequency (%)	Average colonization (%)	Average No. mycorrhizae	Time (months)
CLC 2199	*Suillus* sp. (veil)	16.7	<1	0.7	9
CLC 2341	*Suillus subalpinus*	25.0	<1	0.3	9
CLC 2344	*Suillus variegatus*	16.7	0 – 25	19.7	6
CLC 2345a	*Suillus sibiricus*	0.0	0	0.0	9
CLC 2345a	*Suillus sibiricus*	16.7	<1	0.2	10
CLC 2345	*Suillus sibiricus* 3	0.0	0	0.0	6
CLC 2345	*Suillus sibiricus* 3	40.0	<1	1.2	9
CLC 2345b	*Suillus sibiricus*	100.0	0 – 25	38.9	9
CLC 2345b	*Suillus sibiricus*	100.0	25 – 50	47.0	10
CLC 2294	*Rhizopogon subbadius*	33.3	0 – 25	22.3	6
CLC 2294	*Rhizopogon subbadius*	16.7	<1	6.5	9
CLC 2294	*Rhizopogon subbadius*	16.7	<1	0.3	9
CLC 2294	*Rhizopogon subbadius*	33.3	0 – 25	7.2	10
VT 1009	*Cenococcum geophilum*	16.7	<1	0.8	9
Control	Control	0.0	0	0.0	9

Method 2: Soil inoculum 2 (liquid) & seedlings grown in Styrofoam® blocks (in peat:sawdust).

Isolate Number	Fungus	Colonization frequency (%)	Average colonization (%)	Average No. mycorrhizae	Time (months)
CLC 2035	*Rhizopogon subpurpurascens*	16.7	<1	4.0	9
CLC 2199	*Suillus* sp. (veil)	100.0	25 - 50	47.5	9
CLC 2341	*Suillus subalpinus*	60.0	0 - 25	37.8	9
CLC 2344	*Suillus variegatus*	25.0	0 - 25	48.0	9
CLC 2345	*Suillus sibiricus* 3	0.0	0	0.0	9
CLC 2294	*Rhizopogon subbadius*	0.0	0	0.0	9

Method 3: Spore inoculum & seedlings grown in soil mix 2 in Ray Leach single cell containers.

Isolate Number	Fungus	Colonization frequency (%)	Average colonization (%)	Average No. mycorrhizae	Time (months)
CLC 2375	*Suillus sibiricus*	100.0	25 - 50	49.0	5
CLC 2377	*Rhizopogon subpurpascans*	100.0	25 - 50	30.0	5
CLC 2379	*Rhizopogon* cf *evadens*	100.0	0 - 25	6.0	5
CLC 2380a	*Rhizopogon* cf *molligleba*	100.0	25 - 50	33.7	5
CLC 2381	*Rhizopogon* cf *olivaceofusca*	100.0	25 - 50	59.3	5

Method 4: Soil inoculum 1 (agar plugs) & seedlings in soil mix 1 in Ray Leach single cell containers.

Isolate Number	Fungus	Colonization frequency (%)	Average colonization (%)	Average No. mycorrhizae	Time (months)
CLC 2035	*Rhizopogon subpurpurascens*	0.0	0	0.0	9
CLC 2199	*Suillus* sp. (veil)	0.0	0	0.0	9
CLC 2341	*Suillus subalpinus*	0.0	0	0.0	9
CLC 2344	*Suillus variegatus*	0.0	0	0.0	9
CLC 2345	*Suillus sibiricus* 3	16.7	<1	0.5	9
CLC 2294	*Rhizopogon subbadius*	0.0	0	0.0	9
VT 1009	*Cenococcum geophilum*	0.0	0	0.0	9

The spore method produced the highest colonization rate in the shortest time period for all fungi tested. The pros and cons of using spore inoculum are discussed under conclusions. There was a fungal effect with strains of *Suillus* out performing other groups except when spores were used as an inoculum (Method 3). With spores *Rhizopogon* was able to colonize seedlings at acceptable rates. It was also apparent that substrate type is important as no mycorrhization occurred in soil mix 1 (Method 4) and this concurs with results for other trials using this soil mix (not reported here).

For three selected fungal strains, a 'fungus' effect is evident with high colonization by *S. sibericus* strain (CLC 2345b) and minimal colonization for other strains (Table 3). Light application of fertilizer does not appear to negatively affect colonization except perhaps for *R. subbadius* and may have stimulated colonization for *S. sibericus* CLC 2345b. Seedlings that were well-colonized with CLC 2354b exhibited a darker green needle color (obvious to the observer) and an increase in root development more pronounced with fertilizer. Effects of other fungal strains on seedlings are not discussed due to minimal colonization levels.

Table 3. Effect of light fertilizer treatments (NPK 20-20-20 at 25 ppm) for selected native mycorrhizal fungi on colonization and development of whitebark pine seedlings.

			***Suillus sibiricus* (CLC 2345a)**				
Treatment	**Time (months)**	**Colonization frequency (%)**	**% root colonization**	**No. of mycorrhizae**	**Root development**	**Shoot development**	**Needle color**
1:+ M; + F	10	16.7	0.1 a	0.17 a	1.50 ab	1.67 a	4.33 a
2:+ M; - F	10	16.7	0.1 a	1.17 a	1.83 ab	1.83 a	4.25 a
3: - M; + F	10	0.0	0 a	0.00 a	1.33 b	1.17 a	3.92 a
4: - M: - F	10	0.0	0 a	0.00 a	1.67 ab	1.17 a	4.00 a

			***Suillus sibiricus* (CLC 2345b)**				
Treatment	**Time (months)**	**Colonization frequency (%)**	**% root colonization**	**No. of mycorrhizae**	**Root development**	**Shoot development**	**Needle color**
1:+ M; + F	10	100.0	50-75 a	65.33 a	2.67 a	2.83 a	4.92 a
2:+ M; - F	10	100.0	25-50 b	47.00 a	2.33 a	2.33 ab	4.83 ab
3: - M; + F	-	na	na	na	na	na	na
4: - M: - F	10	0.0	0 c	0.00 b	2.00 a	2.33 ab	4.42 bc

			***Rhizopogon subbadius* (CLC 2294)**				
Treatment	**Time (months)**	**Colonization frequency (%)**	**% root colonization**	**No. of mycorrhizae**	**Root development**	**Shoot development**	**Needle color**
1:+ M; + F	10	0.0	0 b	0.00 a	2.17 a	2.00 a	4.50 ab
2:+ M; - F	10	33.3	0-25 a	7.17 a	1.50 ab	1.33 a	4.17 ab
3: - M; + F	10	0.0	0 b	0.00 a	1.50 ab	1.50 a	4.58 a
4: - M: - F	10	0.0	0 b	0.00 a	1.33 b	1.50 a	3.83 b

CONCLUSIONS

The main goal of this project was to initiate development of methods 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 difficult task) and screening them for potential as inoculum for whitebark pine seedlings. This is an important step since commercial inoculum has the potential to upset sensitive whitebark pine systems and should not be used. Successful mycorrhization occurred in the greenhouse with certain fungi and for particular methods. Therefore, this research was effective in initiating this avenue of research. Next are trials to refine methods for consistent and reliable mycorrhizal colonization on a larger scale.

FUNGAL EFFECTS

In trials using soil inoculum, there was a strong fungal effect with two strains of *Suillus* out-performing other fungi. For the spore slurries, the fungal effect was dampened since all fungi (several *Rhizopogon* and *Suillus* species) tested formed mycorrhizae on 100% of seedlings at various colonization levels. Since fungi are adapted to particular soil types, we continue to screen additional strains. We need to be careful not to select on nursery conditions alone.

GENERAL METHOD & INOCULATION TYPES

A variety of methods (4) with confounding variables were tested as a starting point. Methods 1 and 2 used soil inoculum on seedlings in Styrofoam blocks and have potential for use in nurseries. Increasing colonization rates may depend on improved preparation of inoculum and its use at optimum viability time. Mixing soil inoculum into the substrate when possible is likely to improve colonization significantly, but this may not be feasible under most nursery situations. Liquid inoculum appears to have the potential to increase colonization, but has drawbacks including complex methods prone to contamination. The benefit of using a soil inoculum is that it contains only the fungus of interest, is pathogen free, and may be subsequently generated in the nursery. Subsequent trials will test direct use of liquid inoculum without a ‘soil stage’.

Spore slurries were the most effective method tested resulting in 100% colonization of all seedlings inoculated with suilloids. This method is simple and spores can easily be directly added to seedlings in blocks or 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 that 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. These high elevations sites are prone to drought which prevents fungal fruiting. Inoculation would likely be necessary in spring in the greenhouse and not fall. We are currently testing shelf life for spore slurries and additional methods of storage for spores. In addition, spore slurries are not guaranteed to be free of other fungi, depending on the species used. We are working on methods to reduce or eliminate extraneous organisms.

SUBSTRATE EFFECTS

There is a concern that certain types of substrate are not amenable to mycorrhizal colonization. Soil mix 1 appeared to preclude effective colonization, however colonization occurred in both soil mix 2 and the original mix of peat moss and sawdust in the Styrofoam blocks. New mixes used for seedlings need to be tested before mass inoculation. The Sunshine Mix used in Soil Mix 1 appears to be fungal suppressive as confirmed by other research.

FERTILIZER EFFECTS

There appeared to be no negative effects for the fertilizer levels used, however the fertilizer regime was very light. Some types of fertilizer can prevent mycorrhizal colonization at higher

levels. For CLC 2345b, fertilization appeared to stimulate colonization and this could be a result of increased root development. The effect of higher levels of fertilization on colonization will be tested in subsequent trials.

OVERVIEW

This project discovered several strains of native mycorrhizal fungi that are able to colonize whitebark pine seedlings in the greenhouse. Well-colonized seedlings were dark green with a well-developed root system (with and without fertilizer). Colonization did not increase shoot development (although seedlings appeared more vigorous) and we did not expect this since there is also an initial carbon drain to the fungi. Many of the colonized seedlings showed actively growing shoots in contrast to stagnation with brown buds for controls.

Future trials will be based on this data to test additional fungi, stronger fertilizer regimes, additional substrates and to refine methods. The next goal is to develop a 'reliable' method for mycorrhization of whitebark pine seedlings. Colonization was 'patchy' within treatments; therefore we need to refine methods in order to guarantee consistent mycorrhizal colonization under greenhouse conditions. The time frame also needs to be shortened so that colonization occurs quickly and throughout the root system, and methods need to be realistic and cost effective for the nursery. The drawbacks of spore slurries versus soil inoculum need to be addressed. We are currently moving forward with more trials using colonized seedlings transplanted into biodegradable and plastic pots, testing mycorrhizal colonization after cold treatment, evaluating storage methods for spore slurries and for mycelial inoculum. To date seedlings appear disease free. Strains of nursery fungi (E-strain, *Thelephora*) did not preclude colonization by native fungi and were mostly prevalent when native colonization was absent.

Commercial inocula should not be used in sensitive whitebark pine systems for several reasons. Most commercial inocula do not contain fungi applicable/native to whitebark pine systems (waste of resources), some could promote competitor tree species, and the introduction of alien fungi is of particular concern for National Parks and wilderness areas. In addition, use of non-native fungi risks upsetting the food chain in these forests since local mammals depend on specific mycorrhizal fungi for food and also disperse their spores (Ashkannejhad and Horton 2005, Izzo et al. 2005). Therefore, it is imperative to use regionally-appropriate native mycorrhizal fungi for inoculation of nursery grown whitebark pine seedlings when inoculation is deemed necessary (Wiensczyk et al. 2002).

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Fig. 1. Flow chart for inoculation of native ectomycorrhizal fungi onto whitebark pine seedlings.
Fig. 2. Whitebark pine seedlings with a) chlorotic non-inoculated control on left and seedlings inoculated with b) *Suillus* and c) *Rhizopogon* on the right side.

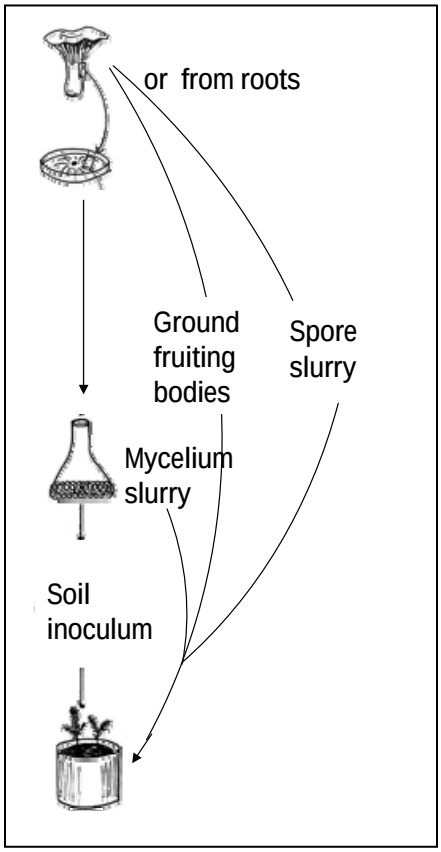

Fig. 3. Mycorrhizae synthesized on whitebark pine seedlings in the greenhouse with a) *Rhizopogon subbadius* b) *Suillus sibiricus* and c) young mycorrhizae of *Suillus sibiricus* on roots.