FINAL REPORT for 2008

Project Status: The project is complete for 2008 and for reporting purposes and for our 2008 proposal; research on inoculation of whitebark pine seedlings is ongoing at MSU. One paper resulted from this research.

Original time line is given first:

JAN 2008: About 16 species of native ectomycorrhizal fungi were grown out (COMPLETED). FEB/MARCH: Seedlings grown at a USDA FS nursery were transferred to MSU (COMPLETED in late March).Additional germinated seed obtained was planted in MSU mix soils in containers (COMPLETED).

FEB/MAR:Screening of mycorrhizal fungi for subsequent testing according to growth rates (COMPLETED). Three species are selected for further testing.

MAR/APRIL: Seedlings for various treatments were inoculated in MSU greenhouse (COMPLETED). MAY-JULY: Seedlings were maintained under greenhouse conditions at MSU Plant Growth Center (COMPLETED).

AUG-SEPT. Fresh sporocarps of native ectomycorrhizal fungi were collected for immediate use in spore slurries and additional seedlings were inoculated. Previously inoculated seedlings were assessed for mycorrhizal colonization. We determined that seedlings needed more time before a complete assessment. There was a delay of mycorrhization.

JAN-MARCH-: Analysis of results on the colonization of whitebark pine seedlings with native mycorrhizal fungi in the greenhouse was completed. We are using a non-destructive method of analysis so that inoculated seedlings can be used for additional tests, and perhaps for out-planting. Bio-containers arrived from Germany and we are using them for transplant tests.

Results:

Overview/abstract: 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 and these are tagged for subsequent trials; 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 under conclusions.

Details

Of the 26 strains of native ectomycorrhizal fungi collected from whitebark pine forests in the Greater Yellowstone Ecosystem (GYE) for initial screening, 16 were grown *in vitro* (Table 1). Six showed vigorous growth and were developed into a soil inoculum and added to seedlings. An additional eight fungi were ground into spore slurries and added directly to seedlings; these were primarily over-ripe suilloid fungi not suitable for tissue culturing. Seedlings were maintained in the greenhouse for several months before assessment.

While it was not possible to test all methods for all fungal strains, 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 these methods (1 &2). The spore method produced the highest colonization rate in the shortest time period for all fungi tested. 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. Also apparent, is 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 was 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 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.

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	9
VT 1009	*Cenococcum geophilum*	0.0	0	0.0	9

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 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 the spring under greenhouse conditions and not fall. So we are currently testing shelf life for spore slurries and additional methods of storage for spores. In addition, spore slurries are not always 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 fungi. Well-colonized seedlings also exhibited more actively growing shoots and less stagnation (brown buds).

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). Therefore, it is imperative to use regionally-appropriate native mycorrhizal fungi for inoculation of nursery grown whitebark pine seedlings when inoculation is deemed necessary.

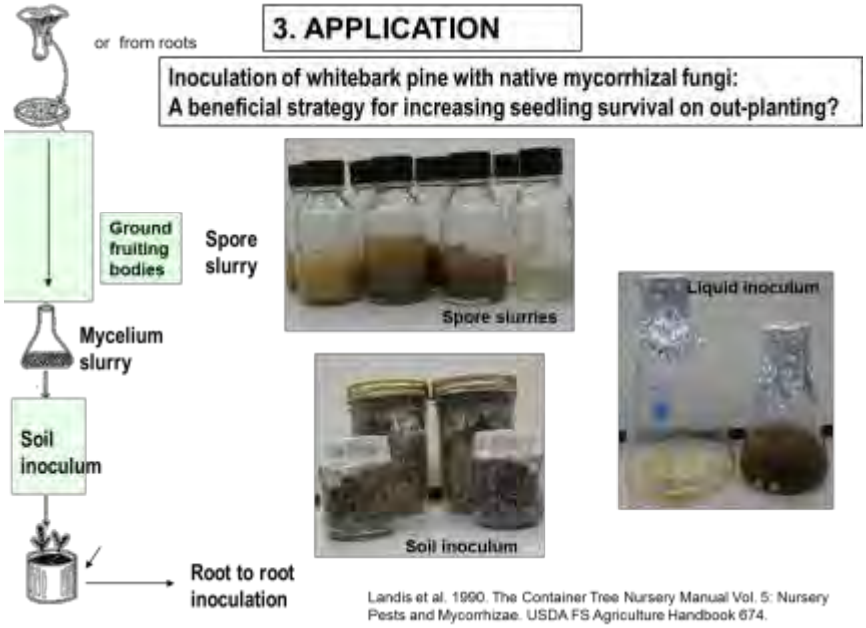
Changes needed or Problems Encountered:
It appears that some substrates may suppress efficient mycorrhization and we adjusted for this. Mycorrhizal colonization took longer than expected and we are working to shorten this time frame.

Sharing Results/Products/Outcomes:
We previously attached our Powerpoint presentation and the handouts given out at the WIFDC meeting.

Suggestions for how the overall program can be improved to better meet your needs: We appreciate allowing us to first give a progress report followed by this final report, since whitebark pine have slow growth and experiments take time. Continued funding *will be essential* for this avenue of research and hope it is available as personnel decisions need to be made soon for 2009. We also appreciate the interaction with the Forest Nursery in Idaho as a source of seedlings.

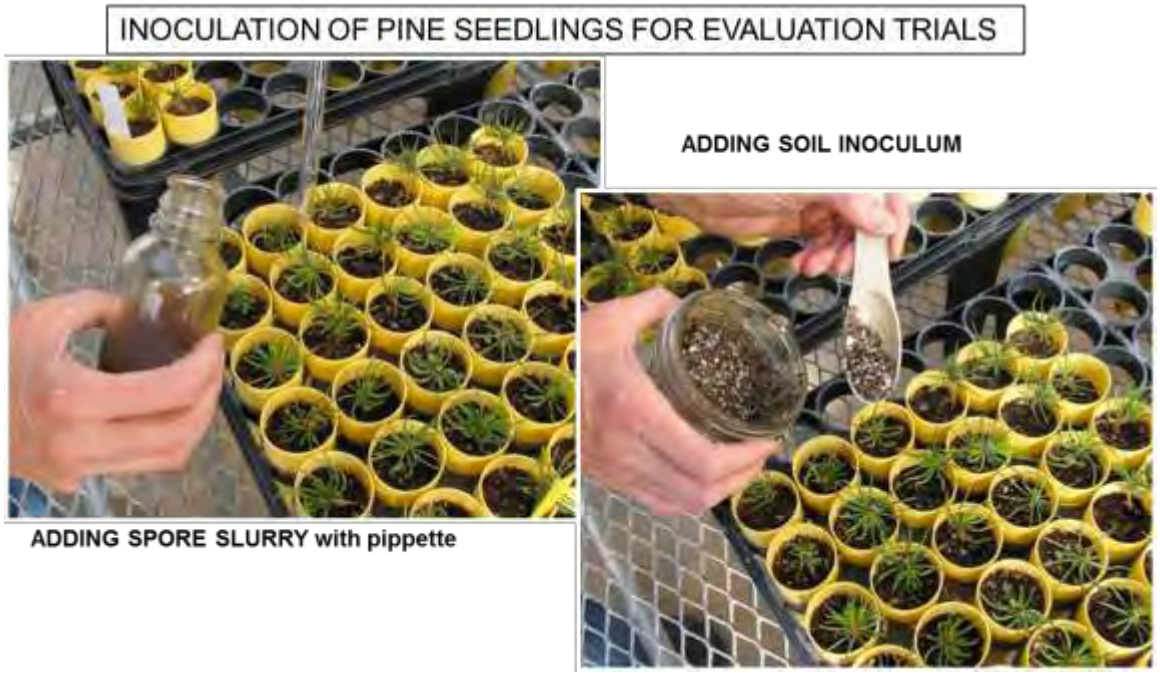
1. Ectomycorrhizal fungi are known to enhance seedling establishment in outplantings. First we needed to know what fungi were found with whitebark pine. Fruiting bodies of beneficial ectomycorrhizal fungi specific to whitebark pine were collected in the Northern Rocky Mountains under whitebark pine.

2. Inoculum was developed from these fungi by various methods—liquid inoculum, soil inoculum, and spore slurry inoculum were tested.

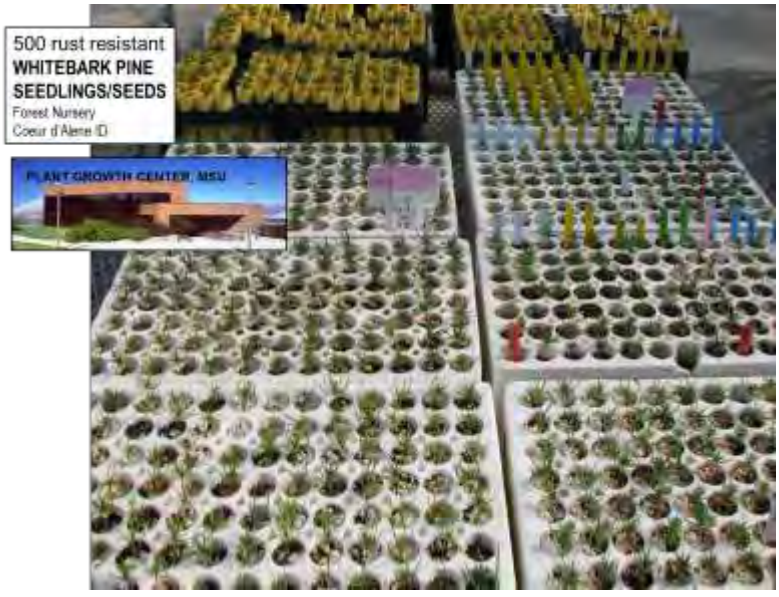


Landis et al. 1990. The Container Tree Nursery Manual Vol. 5: Nursery Pests and Mycorrhizae. USDA FS Agriculture Handbook 674.

3. Various types of mycorrhizal inoculum was added to seedlings.



4. Seedlings from the Coeur d'Alene nursery were used in the experiment and transplanted at the MSU Plant Growth Center.



5. Seedlings were inoculated and several variables were tested over the next few months.

EXPERIMENTAL DESIGN --variables

- rust resistant seedlings
  - styrofoam blocks
  - conetainers
  - age of seedlings at inoculation
- species/isolates of fungi
  - screening
  - spore slurries (fresh)
  - spore slurries (aged)
  - mycelium liquid
  - soil inoculum
  - +/- fertilizer
- inoculation in the nursery
  - before/after transplanting to larger pots
- transplant potting media types
  - peat
  - sawdust
  - soil types
  - suppressive soils?

Roots of a containerized whitebark pine seedlings colonized with a Suilloid ectomycorrhizal fungi.

6. A summary of our results:

- **we found 32 species of ectomycorrhizal fungi with whitebark pine**
- **a majority of the 25 isolates tested could successfully colonize whitebark pine seedlings and the most efficient strains were selected for further testing (Suillus, Rhizopogon = suilloids)**
- **all types of inoculum resulted in mycorrhizal colonization, but spore slurries were the most efficient in terms of labor and time**
- **certain types of soil were surpressive to mycorrhizal colonization**
- **certain types of fertilization could affect mycorrhizal colonization, but the fertilization regime was light for these trials and needs further testing**

Papers resulting from this research and a link to each paper:

Cripps, C.L. and R. Antibus. 2011. Native Ectomycorrhizal fungi of limber and whitebark pine: necessary for sustainability? Pgs. 37-44. In: Keane, R. et al., editors, 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; USDA FS, Rocky Mountain Research Station.

Cripps, C.L. and Eva Grimme. 2011. Inoculation and successful colonization of whitebark pine seedlings with native ectomycorrhizal fungi under greenhouse conditions. Pp. 312-322. In: Keane, R. et al., editors, 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; USDA FS, Rocky Mountain Research Station.