USDA United States Department of Agriculture | Forest Service | Region One | Northern Region 200 East Broadway Missoula, MT 59802

2011 WHITEBARK PINE RESTORATION PROJECT PROGRESS REPORT

Submit the following information electronically to John Schwandt (via Candee Wilfong at CWilfong@fs.fed.us) *by 1/18/2012*

Project Title: Inoculation of whitebark pine seeds and seedlings with spores of native ectomycorrhizal fungi

Project Contact: Dr. Cathy L. Cripps, Plant Sciences & Plant Pathology Dept., Montana State University, Bozeman, MT 59717, 406-994-5226, ccripps@montana.edu

Location: (state, agency unit, etc.) MSU Plant Growth Center (Bozeman, MT) and areas selected to be direct seeded with whitebark pine seeds (Yellowstone Club, Idaho, etc.)

Size of Treated Area: N/A

Reported in FACTS (if applicable)? (N/na)

Objective(s) (from original request)**:**

Objective(s): Previous objectives we have met in Phase I and 2:

√1. Determine the ectomycorrhizal fungi important to *Pinus albicaulis* on a regional scale.
√2. Isolate, cultivate and maintain cultures of native ectomycorrhizal fungi with whitebark pine.
√3. Screen for native fungi that grow at rates sufficient for inoculation of nursery seedlings.
√4. Develop a soil inoculum of selected native ectomycorrhizal fungi on a small scale.
√5. Develop protocol for mycorrhization of nursery seedlings with native fungi *and in progress*.

The objective of the current project is to examine and develop various methods for the application of ectomycorrhizal basidiospores (spore powder, spray slurry, chips) to whitebark pine seeds that are to be directly planted in the field. The goal is enhanced germination, enhanced survival and/or enhanced/timely mycorrhizal colonization of germinants inoculated with native ectomycorrhizal fungi specific to 5-needle pines. We are also doing additional work on inoculation of nursery seedlings.

Budget	**Requested Funding $**	**Other Funding $**	**Description /Source/in-kind** Please list origin of matching funds
Salary	9,000	2,600	Salary for technician/student
Travel	800		Travel to
Contracting			
Equipment			
Supplies	2,800	700	Greenhouse rental, supplies
Other (specify)			
Totals	12,600	3,300	Salary for technician/student

Did FHP funding get used or obligated; if not briefly explain

Project Status: This project is ongoing and grant ends in 2013. This is a progress report.
Results: (what did you accomplish and what have you learned)

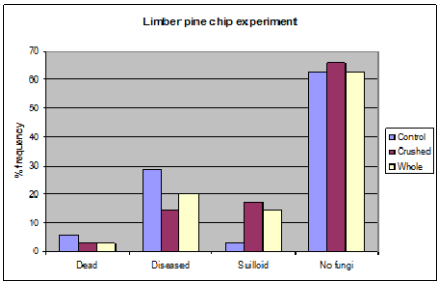
We have several ongoing experiments for which we are waiting for final results and two completed experiments.

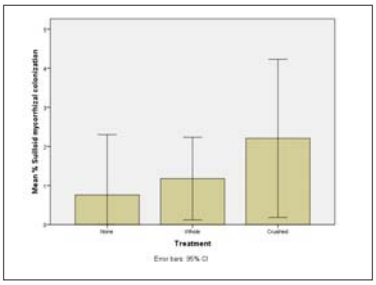
1. Development of Basidiospore chips: the goal of this research is to develop a 'chip' type of inoculum that can be added to whitebark pine seedlings or seeds at the time of out-planting.

Spores of Suillus sibiricus (known to form mycorrhizae with 5-needle pines in the greenhouse) taken from dried sporocarps were made into a slurry with distilled water. Spores were counted using a hemocytometer and the slurry was further diluted to a spore count of approximately 2 x 10^6 spores/ml.Basidiospore chips (basidiochips) were prepared by thoroughly mixing: 20 ml autoclaved peat moss sifted through a 5 mesh sieve, 20 ml J-tac (a hydrocolloid used in reclamation), 400 ml autoclaved sand, 60 ml spore slurry, and ~10 ml distilled water until mixture felt pasty. The mixture was then rolled out on a metal sheet to a thickness of 2mm and cut into 2 cm^2 chips. Basidiochips were covered and air dried for 48 hours before being lifted with a spatula, bagged, and stored in a refrigerator. Each basidiochip contained approximately 200,000 spores and each seedling received 3 chips for a total of 600,000 spores per seedling.

One hundred and forty five limber pine seeds were obtained from a Colorado nursery. Seeds were rinsed for 24 hours and cold stratified for 30 days. Limber pine seeds were used in initial trials so that whitebark pine seeds could be saved for subsequent trials. For stratification, seedlings were placed in the refrigerator in plastic sandwich bags containing perlite and were not rinsed. Seeds were then planted directly into short Ray Leach cone-tainers (3.8 cm x 14 cm) and maintained under standard greenhouse conditions. The 105 emerged seedlings were transplanted into Soil Mix 3 and replanted into Stuewe & Sons short Deepots (2.5cm x 12.7cm) and maintained under standard nursery conditions for 9 months. Seedlings were vernalized in a cold room of approximately 4^{0}C for 1 month and then randomly separated into one of three treatment groups: 35 seedlings received 3 whole basidiochips, 35 seedlings received 3 crushed basidiochips, and 35 seedlings received no basidiochips as a control.

Results showed a very low colonization and unacceptable mycorrhizal colonization rate using the basidiospore chips. This could be a result of the hydrocolloid component which might have desiccated or attached to spores. Alternatively, the dried inoculum might have been ineffective before being used in the basidiospore chips. This second hypothesis is currently being tested by using the spores without the other 'chip' components. If spores are otherwise viable, then the "basidiospore chip" line of research will be terminated.





Figures show the low colonization rate

2. Inoculation (using liquid slurry) of directly planted seeds after warm stratification and prior to vernalization. This experiment was intended to mimic the direct out-planting of warm stratified seeds in Fall. [This experiment also tests viability of spores used in basidiospore chip experiment]

Warm stratification: One hundred whitebark pine seeds were obtained from J. Schwandt. Seeds were placed in two mesh bags and soaked in a running water rinse for approximately 48 hours. Mesh bags containing seeds were placed inside 1-mil polyethylene bags partially filled with vermiculite. The tops of the polyethylene bags were left open to allow air circulation. Seeds were then warm stratified at a constant temperature of 22^o C for 4 weeks with no photoperiod. Once every week mesh bags were removed from polyethylene bags and soaked in a running water rinse for one hour. Seeds were checked daily for signs of disease or germination; seeds that appeared moldy or germinated were removed.

In order to mimic fall direct seed planting methods developed by John Schwandt, following the 4-week warm stratification seeds were directly planted into chilled soil and placed in a cold room maintained at approximately 4 ^{o} C instead of undergoing a 60-90 day cold stratification as is standard practice. At the time of planting, seeds were randomly split into mycorrhizal treatment groups and received an ectomycorrhizal spore powder, spore slurry, or no mycorrhizal treatment. All seeds were planted at a depth of approximately 2 cm.

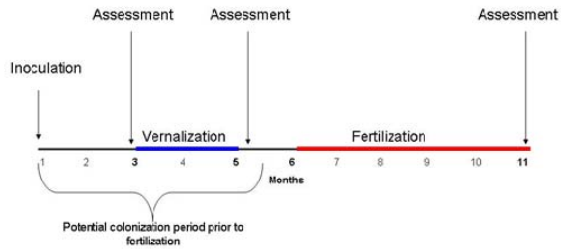
Spore Powder: was made from frozen Suillus sibiricus (CLC 2440-2009). The hymenium was removed and cut into small pieces before being frozen. Frozen hymenium was then ground into a coarse powder using a coffee grinder and the spore content per gram was determined using a hemocytometer. Each of the seeds randomly assigned to the spore powder treatment group received 0.1g of spore powder containing approximately 2 x 10^6 spores.

Spore Slurries: were made from two collections of Suillus sibiricus, (CLC 2440-2009) and (CLC 2779-2011). The hymenium was processed as above and the resulting material was strained into 400 ml of sterile distilled water and stored in glass bottles in the refrigerator. The spore content of both slurries was then counted using a hemocytometer and diluted to 1x 10^6 spores/ml. Seeds in both spore slurry treatment groups were inoculated using an Allflex 50mL repeat syringe and received 2 ml of spore slurry for a total of 2 x 10^6 spores. Seedlings in the powder and control treatment groups received 2 ml of distilled water to ensure moisture levels in all treatments remained approximately equal.
Results for this experiment are pending.

Number of seeds	Treatment	Fungus
24	Powder	CLC 2440
24	Slurry	CLC 2440
24	Slurry	CLC 2779
23	Control	NA

3. This large experiment is to determine if seedlings already colonized with mycorrhizal fungi can then be subjected to a final fertilization treatment (before out-planting). The goal is to determine if the mycorrhizal condition is maintained after the fertilization treatment. This would allow for both mycorrhizal inoculation and fertilization before out-planting.

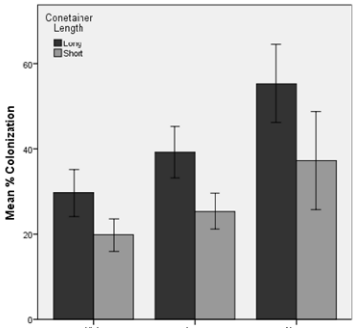
Below is a timeline showing inoculation of seedlings with mycorrhizal fungi followed by vernalization and then fertilization. Assessments refer to examining seedlings for mycorrhizal colonization.Seedlings were not fertilized 1 month before inoculation or during vernalization so that mycorrhizal colonization could take place. Two levels of (low nitrogen) fertilization were used and other variables such as container length, type of slurry, and method of inoculation were also examined. Suillus sibiricus was again used as the native mycorrhizal fungi in the inoculum types.



Container Length	Slurry Type	Inoculation method	High Fertilizer	Low Fertilizer	No Fertilizer
Long	Fresh	Drip	24	23	10
		Inject	25	24	10
	Dried	Drip	21	21	10
		Inject	23	23	10
	NA	NA	18	17	0
Short	Fresh	Drip	28	28	10
		Inject	26	26	10
	Dried	Drip	24	23	10
		Inject	24	23	10
	NA	NA	6	7	0

Briefly, results for this experiment showed no effects for slurry type or inoculation method so data was pooled for the graph shown below to highlight the effects of fertilization and container length on mycorrhizal colonization of seedlings. .

Results showed that mycorrhizal colonization was maintained after both the high (once a week) and low (once every other week) fertilization using a low nitrogen Fertilizer (Phosgard 4-25-15 liquid). *However, fertilization did reduce the level of mycorrhizal colonization in comparison to controls. A next step might be to determine how mycorrhizal colonization versus nitrogen-addition affects N-foliar content in needles to determine if N levels from inoculation can be comparable to those found after final addition of nitrogen fertilizer. Results show that it is worth exploring the level, type and timing of fertilization in order to allow and maintain mycorrhizal colonization.*



Also, short cone-tainers were tested because it is often difficult to plant long root systems into shallow, rocky soils. While the short cone-tainers (Ray Leach cone-tainers (3.8 cm x 14 cm) did produce a rather robust root system when well-colonized with mycorrhizal fungi, there was apparently some sensitivity to watering and fertilization regimes which might have been too high for the shorter cone-tainers. Long cone-tainers were Ray Leach cone-tainers (3.8 cm x 21 cm) and we do believe it would be worth exploring short cone-tainers 16-18 cm in length.

4. We were involved with a direct seed planting experiment at the Yellowstone Club and Fairy Lake directed by J. Schwandt.

I was involved with the set up of the experiment at the Yellowstone Club on Sept 21, 2010. My graduate student assisted John Schwandt and Holly Kearns with data collection for whitebark pine direct seeding trials at Fairy Lake on 8/1/2011 and at the Yellowstone club on 8/2/2011. Two treatments involved direct seed planting in conjunction with addition of "spore powder" of Suillus sibiricus developed into an inoculum for seeds. Results are pending from Schwandt et al.

Changes needed or Problems Encountered:

Research is continuing and progressing; the main problem is accessing seeds or seedlings for our research since we do not collect cones.

Sharing Results/Products/Outcomes: (If applicable, please attach or send reports, photos, presentations, websites, etc.)

Recommendations for inoculation of whitebark pine seedlings with native fungi are included in the Range-wide Restoration Strategy for Whitebark pine (Keane et al, in ed, to be published in 2011). One paper on results for the greenhouse fertilization experiment is underway. On presentation on this information was given at the Whitebark Pine Foundation meeting in Cody, Wyoming.

Suggestions for how the overall program can be improved to better meet your needs: (suggestions regarding RFP solicitation and evaluation process, etc.)

We would like to obtain older seedlings (about 16 mo old) for inoculation. A small previous experiment showed that a typical fertilization regime could proceed for 16 mon, and that seedlings could then be inoculated a few months before out-planting (and then without fertilizer) with excellent colonization results. We would like to try this again on a larger scale.

We have other experiments underway that are not reported here.

Additional figures for projects

Suilloid mycorrhizae produced in the greenhouse on whitebark pine seedlings.

Experiment to determine if fertilization and mycorrhizal colonization can co-occur.

Basidiospore chips experiment to determine if chips containing 1 million spores each of native mycorrhizal fungi can be used to inoculate seedlings. In the field, chips would be buried with the seedling root system..