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INFLUENCE OF PISOLITHUS TINCTORIUS INOCULATION ON GREENHOUSE GROWTH AND FIRST-YEAR TRANSPLANT SURVIVAL OF CONIFER SEEDLINGS[1]

By

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Abstract: Mycorrhizal fungi form a symbiotic association with the root systems of most higher plants. Mycorrhizae colonization of root systems is believed to improve tolerance to adverse soil conditions such as low pH or high salinity. Mined land reclamation may require transplanting seedlings onto harsh sites that may have low pHs, high salinity, low nutrient status, etc. The purpose of this study was to examine whether inoculation of conifer seedlings in the greenhouse with *Pisolithus tinctorius* would improve first year survival of seedlings transplanted onto overburden material at the Molycorp Questa Mine in northern New Mexico. Seedlings of *Pinus ponderosa, P. edulis, P. strobiformis, P. flexilis, P. aristata, P. sylvestris, and P. nigra* were used in this study. Subsets of each species were inoculated with *Pisolithus tinctorius* at either six or ten weeks after germination or not artificially inoculated. Seedlings were evaluated for growth response in the greenhouse after inoculation and before transplanting. Inoculation and growth media composition significantly impacted shoot height and caliper growth but responses were species dependent and the magnitude of the differences between inoculated and non-inoculated seedlings were small. Seedlings were transplanted in August 1996 on a site at an elevation of 9,500 ft. and with substrate pHs ranging from 3.5 to 4.0. The impact of inoculation with *Pisolithus tinctorius* on survival was variable by species. Only *P. strobiformis* had improved survival with inoculation (>20%).

Additional Key Words: acid soils, disturbed land reclamation, reforestation

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Introduction

The significance of mycorrhizae on higher plant – environment interactions is well known. Ectomycorrhizae, those mycorrhizae which do not penetrate root cells, are commonly found in association with coniferous species including members of the genus Pinus. It has been estimated that there are over 5000 species of ectomycorrhizae fungi world wide (Marx 1991). Benefits of mycorrhizal associations to plants used in reforestation and revegetation may include improved survival (Marx 1991, Marx et al. 1992, Marx and Cordell 1989), enhanced growth (Browning and Whitney 1992), improved tolerance to water deficits (Boyle and Hellenbrand 1991; Svenson et al. 1991), and superior performance on low nutrient sites (Marx 1991). However, these responses are often species, host and fungal species, specific as well as site specific.

There is a cost to the host plant along with the benefits of the association. Mycorrhizal fungi, like all fungi, are heterotrophic organisms which depend on an external source of organic carbon for their energy needs. In ectomycorrhizal plants it is estimated that as much as 24% of the total carbon assimilated is allocated or used by the fungal symbiont (Vogt et al. 1982). More frequently, estimates of this cost to the host plant range from 15 to 20% of the total carbon assimilated (Soderstrom 1991). A considerable amount of work has been done examining the impact of this "cost" in producing container reforestation and revegetation planting stock (St. John and Evans 1990; Marx 1991). In general, alterations, primarily in fertility regimes, are required when producing stock with good mycorrhizae colonization. Marx (1991), summarizing the state of research at the time, concluded that any condition which impacts carbon allocation to roots will impact ectomycorrhizal development. The challenge is to develop a seedling production regime in which the end product has the desirable or target morphological and physiological attributes, including sufficient mycorrhizal development, to meet planting needs.

The mine site used (Questa molybdenum mine) in this study is located in the Taos Range of the Sangre de Cristo mountains in northern New Mexico. Elevation at the site ranges from **2,400 to 3,000 m** (8,000 to 10,000 feet). The terrain surrounding the mine supports primarily coniferous ecosystems with riparian ecosystems in the bottoms of many canyons having perennial streams or rivers. The conifer ecosystems include ponderosa pine (*Pinus ponderosa*), mixed conifer (*P. flexilis, Pseudotsuga menziesii,*

Abies concolor) to spruce-fir (*Picea engelmannii* and *A. concolor*) stands. Distribution of these species is influenced by topographic features as well as aspect.

Open pit mining operations were conducted between 1965 and 1983. During this time approximately **300 metric (328) million** tons of waste rock were produced and placed in rock piles surrounding the open pit. In general, mixed volcanic waste rock was excavated from a hydrothermal scar area of the pit (SRK 1995). These mixed volcanic rocks were derived from upper rhyolitic and lower andesitic series rocks of Tertiary age. The mixed volcanics are highly fractured and weathered, and typically exhibit a paste pH in the range of 2.3 to greater than 6.0, the majority less than 3.5. Paste extractions of these rocks typically indicate a high TDS content. The remainder of the waste rock was derived from propylitic black andesite, aplite and granite. Black andesite, aplite and granitic intrusives (mine aplite) typically exhibit neutral paste pH and low paste TDS content.

Objectives

The potential for colonizing disturbed sites with soil microorganisms, such as mycorrhizae, via the use of inoculated container grown planting stock is becoming increasingly accepted. Three experiments were conducted to examine the effects of *Pisolithus tinctorius* inoculation on the nursery culture and first-year survival of container grown pines planted directly into overburden at the Molycorp, Inc. Questa mine. The first experiment examined the effect of timing of inoculation on shoot growth and stem caliper during greenhouse culture. The second experiment examined the influence of growth media composition on the same responses evaluated in the first experiment. The third experiment examined the first-year survival of inoculated or non-inoculated seedlings planted on the overburden piles at the Molycorp Inc., Questa mine.

Materials and Methods

Experiment 1.

Six species of pine were used in this study: *Pinus aristata, P. edulis, P. nigra, P. ponderosa, P. strobiformis*, and *P. sylvestris*. Seedlings were grown from seed in a greenhouse under a modified greenhouse production regime in 164 cm^3 containers filled with a 2:1:1 (v:v:v) peat:perlite:vermiculite growing media. General greenhouse conditions included a 16-hour photoperiod (ambient light plus supplemental light from high pressure sodium vapor lamps suspended above the seedlings); day

temperatures ranging from 20 to 27°C, night temperatures ranging from 19 to 23 °C. Seedlings were irrigated as needed. The only fertilizer seedlings received was from a resin-coated, slow release fertilizer (Osmocote, 14-14-14, 3-4 month) incorporated into the media at a rate of 4 kg/ m^3. This reduced fertility regime, relative to the standard seedling production regime, was based on previous studies that found high fertility levels can reduce mycorrhizal colonization of root systems and use of slow release fertilizers has been shown to be less detrimental than water soluble fertilizers (Maronek et al. 1982; Crowley et al. 1986; St. John and Evans 1990).

Three inoculation treatments were used. The first treatment involved inoculating 196 seedlings of each species with a commercial source of *Pisolithus tinctorius* six weeks after germination. Inoculation was accomplished by drenching the root plug with a spore suspension of *P. tinctorius* in water (MycorTree PT Spore Spray, Plant Health Care, Inc., Pittsburgh, PA, USA 1995). The second inoculation treatment was similar to the first, except that inoculation occurred at ten weeks after germination and involved 196 seedlings of each species. The third treatment, the control group, was not artificially inoculated **and** **included** ~~The control group was also replicated by~~ 196 seedlings of each species. At 20 weeks after germination, shoot height from the cotyledon scar to the tip of the growing apex was measured to the nearest 0.5 cm using a ruler. Stem caliper was measured to the nearest 0.1 mm using a digital caliper. Mycorrhizal colonization was determined by removing five to ten seedlings of each inoculation by species treatment combination from their containers and visually inspecting the root plug for presence of mycorrhizal inoculation using a procedure modified from Cordell et al., 1990. Lateral roots growing on the periphery of the root plug were examined for the presence of root bifurcations and fungal hyphae as indicators of the presence of mycorrhizal colonization.

The treatment design was a factorial combination of species (6) by inoculation treatment (3). The experimental design was a completely randomized design with each species x inoculation treatment combination being replicated by 196 seedlings. Growth data was analyzed using analysis of variance (PROC GLM; SAS Inc. 1990). Two analyses were run, the first model tested included species in the model being tested. The second set of analysis was run by species looking at the main effect of inoculation treatment on the two growth attributes.

Experiment 2.

Only *P. ponderosa* seedlings were used to examine the influence of growth media composition on the efficacy of *P. tinctorius* colonization. The three growth media were a 2:1:1 (v:v:v), 1:1:1 or a 1:2:1 mixture of peat:perlite:vermiculite. The three growth media were labeled heavy, medium and light, and had calculated dry bulk densities of approximately 0.126, 0.103 and 0.101 g/cm^3 , respectively. The calculated wet bulk densities for the heavy, medium and light growth media were 0.584, 0.576 and 0.531 g/cm^3 , respectively. Growth media bulk densities were calculated from published component values (Landis et al., 1990). Seedlings were produced and inoculated as described for Experiment 1. The same three inoculation treatments used in Experiment 1 were used in this experiment. Shoot height, stem caliper, and successful mycorrhizal colonization were measured or determined as described in Experiment 1.

The treatment design was a factorial combination of growth media composition (3) by inoculation treatment (3). The experimental design was a completely randomized design with each growth media x inoculation treatment combination being replicated by 196 seedlings. Growth data was analyzed using analysis of variance (PROC GLM; SAS Inc. 1990).

Experiment 3.

Seedlings produced in Experiments 1 and 2 were used in this experiment. In addition, *P. flexilis* seedlings receiving the same treatment combinations as described in Experiment 1 were also used in this experiment. Seven-month old seedlings were planted in late August of 1996 using dibble bars on a bench site at the Molycorp, Inc. mine (Capulin overburden pile). The site had previously been ripped to mitigate compaction problems at the site resulting from the bench being previously used as haulage/dumping area during the open pit operation of the mine. A 45 cm inch ripping depth was targeted, however, actual ripping depth varied from 45 cm to less than 15 cm. The site was also variable in terms of overburden chemical properties (Table 1). Three overburden samples from each block were taken and analyzed for chemical and physical properties. Samples were taken from a depth of **5 cm** to 15 cm at each sampling location. Samples were sent to commercial laboratory for analysis (Energy Laboratories, Inc. Billings, MT, USA). Following planting, seedlings were irrigated by hand with approximately 4 liters of water per seedling. No further irrigation occurred.

The treatment design was a factorial combination of species (9 (7 tree species + 2 additional *P. ponderosa* media composition treatments resulting from experiment 2)) by inoculation treatment (3). The

experimental design was a randomized complete block design with 8 blocks. Each species by inoculation treatment combination was replicated by a 10-tree row plot per block. The response variable, survival, was the percent survival for the 10-tree row plot.

Survival data were first analyzed as a nine (species) by three (mycorrhizal treatment) by eight (block) factorial, and then separately by source. Categorical analysis of variance (SAS Proc **CATMOD?** Catmod, SAS Institute 1990) was used to determine treatment differences using the factorial treatment structures described for each experiment. This procedure is a generalization of the chi-square test of homogeneity, which uses the "logit"--the natural log of the ratio of surviving to non-surviving trees for each treatment combination--as the response variable. Low cell counts made it necessary to use generalized least squares. Observed significance levels less than or equal to 0.05 were considered significant. Percentages and standard errors were calculated for main effects and interaction combinations. Finally, approximate pairwise Z statistics were used to conduct pairwise comparisons of main treatment effects using a conservative alpha value of 0.05 divided by the number of comparisons.

Results and Discussion

The influence of *Pisolithus tinctorius* inoculation on shoot development in the first experiment varied between species with three species (*P. edulis, P. nigra* and *P. ponderosa*) being adversely affected by inoculation (Tables 2, 3, Figures 1 and 2). While inoculation had a statistically significant impact on both shoot attributes (height and caliper) of all species, except caliper size in *P. edulis*, actual differences in shoot sizes were quite small (Figures 1, 2). This resulted in very little variability in overall shoot sizes of treated seedlings used in the third experiment. Similarly, both inoculation and growth media composition impacted both shoot parameters of *P. ponderosa* in the second experiment, though the differences were quite small (Table 4, Table 5). In this experiment, the range of average stem caliper among treatments was less than 1.1 mm. Average seedling size for all the species produced in this study, regardless of inoculation treatment, was smaller than expected. This may have been due, in part, to the **lowered** fertility regime used. Traditionally, these species are produced using a combination of slow release fertilizers and water soluble fertilizers. The fertility regime used in this study relied solely on the slow release fertilizer. The target minimum shoot height for conifer seedlings produced at the Mora Nursery is 15 cm. Average shoot

height of seedlings produced in this study ranged from 14 cm for *P. strobiformis* and *P. sylvestris* to 6.5 cm for the slower growing *P. edulis*.

Both inoculation treatments were successful in colonizing the root systems of all treated seedlings in all six species evaluated. Over 70% of the feeder roots of inoculated seedlings had evidence of mycorrhizal colonization compared to less than 5% of all the feeder roots inspected in the control group. All ***(previous sentence says 70%)*** seedlings in both the six-week and ten-week inoculation treatments had mycorrhizal colonization based on visual inspection. The outer part of the root balls of these seedlings had very pronounced hyphal layers and a considerable amount of root bifurcation. The amounts of both these attributes were considerably greater than is normally observed in the nursery ~~and was seen in the control group~~, indicating that the elevated levels were due to the inoculation treatments. This colonization is in contrast to the control seedlings where the hyphal wefts, if present, were smaller and the frequency of root bifurcation less. Other studies have also found that unless intentionally inoculated, container grown seedling mycorrhizal colonization can be sporadic (Marx 1991). Since, mycorrhizal species was not identified as part of this process we cannot confirm the hyphal wefts and root bifurcation were from the *P. tinctorius* used in the inoculum. However, the scant amount of mycorrhizal roots in the control group or elsewhere in the nursery lead the investigators to conclude the mycorrhizae present in the root systems of inoculated seedlings was the applied *P. tinctorius*.

First year survival was influenced by tree species, blocking, their interaction and the interaction of tree species and inoculation treatment (Table 6). Overall, species survival ranged from 59% for *P. flexilis* to 26% for the *P. ponderosa* grown in the lowest density, 1:2:1 media (Figures 3, 4). Only in two species, *P. nigra*, and *P. strobiformis*, was survival influenced by an inoculation treatment relative to control (Table 7). In *P. strobiformis*, both inoculation treatments improved survival. *P. nigra* seedlings, inoculated at ten weeks after germination, had reduced survival compared to control seedlings and seedlings inoculated six weeks after germination (Figure 3). The results are in contrast to other reported studies where mycorrhizal seedlings had improved survival (Marrs et al. 1999, Cordell et al. 1999). In these studies and reviews, overall improvement in survival ranged from 3% (Davies and Call 1990 as cited by Marrs et al. 1999) to over 35 % (Cordell et al. 1999).

The magnitude of the blocking effect, when analyzed in the complete model or by species, was an overriding factor in the survival response (Figure 5). Survival between blocks ranged from slightly over 8% in block 5 to near 70% in blocks 1 and 2. This may be due to differences in substrate geology (Table 1) and probably more importantly, the depth achieved during the ripping process prior to planting. Other studies have found or implied the deleterious **effect** ~~impact~~ of compaction on seedling survival (Cleveland and Kjelgren 1994; Graves 1999; Vimmerstedt et al., 1999). In field notes at the time of planting it was noted that blocks 5, 6, and 7 all had a high occurrence of shallow ripping (approximately 15 cm). These three blocks also had the lowest first-year survival rates. Others have reported improved survival when treatments such as tillage, ripping or backhoe excavation are used to mitigate the effects of compaction (Cleveland and Kjelgren 1994; Graves 1999; Vimmerstedt et al., 1999). While compaction (blocking) appeared to influence survival, it did not influence the survival response to inoculation (Table 6).

Conclusions

From this work, artificial inoculation of conifer seedlings with mycorrhizae merits further investigation. It is clear that changes to production techniques in the greenhouse are needed in order to produce seedlings meeting target height in the expected time. The benefit of *P. tinctorius* inoculation may be species specific with only one of the seven species having first-year survival improved by inoculation in this study.

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Table 1. Overburden Chemical and Physical Properties for the Experiment 3 Planting Site. Numbers Reflect the Mean of Three Samples Measured for Each Block.

Overburden Parameter	Block							
	1	2	3	4	5	6	7	8
pH[1]	4.60	4.30	4.40	4.23	3.87	3.47	3.20	3.00
Conductivity (mmhos/cm)[1]	0.46	1.55	0.95	1.66	2.42	2.09	5.43	8.15
Base Sat.(%)[1]	19.63	20.93	21.33	24.17	24.37	30.50	26.43	26.40
Calcium (meq/l)[1]	0.97	6.29	3.86	15.07	2.99	2.58	8.98	3.26
Magnesium (meq/l)[1]	0.36	1.02	1.11	1.68	2.92	3.76	6.82	12.25
Sodium (meq/l)[1]	0.49	0.55	0.67	0.46	0.40	0.28	0.45	0.55
S. A. R.[1]	0.67	0.41	0.46	0.27	0.23	0.20	0.30	0.51
Organic Matter (%)	0.29	0.29	0.30	0.25	0.25	0.27	0.22	0.25
Phosphorus (µg/g)[2]	3.87	5.13	7.70	10.70	9.07	23.97	3.50	4.90
Potassium (µg/g)[3]	72.00	65.00	73.33	62.33	51.33	52.67	48.67	67.67
Aluminum (µg/g)[4]	9.93	9.13	8.17	5.70	1.17	3.00	152.77	201.53
Copper (µg/g)[4]	0.70	0.50	0.47	0.60	0.47	0.30	0.57	0.63
Iron (µg/g)[4]	264.33	346.00	308.67	318.00	448.67	403.67	436.00	544.00
Manganese (µg/g)[4]	34.23	12.93	24.17	6.83	5.93	4.57	3.90	4.50
Zinc (µg/g)[4]	1.40	1.23	1.47	0.63	1.43	0.73	2.00	2.13
Molybdenum (µg/g)[5]	0.77	0.80	0.73	0.63	0.77	0.60	0.53	0.57
Physical Parameter								
Sand (%)	82.33	76.67	78.33	73.33	70.33	59.67	69.67	67.00
Silt (%)	9.33	13.33	12.67	13.33	17.67	21.00	19.00	17.67
Clay (%)	8.33	10.00	9.00	13.33	12.00	19.33	11.33	15.33

[1]Saturation Paste Extract

[2]Sodium Bicarbonate Extract

[3]Ammonium Acetate Extract

[4]DTPA

[5]ABDTPA

Table 2. Analysis of Variance Table for *Pisolithus tinctorius* Inoculation Effects on Caliper and Height Growth for Six Pine Species.

		Caliper Growth		Height Growth	
Source	df	MS	Pr > F	MS	Pr > F
Model	17	40.68	0.0001	1746.37	0.0001
Species (S)	5	116.72	0.0001	1040.01	0.0001
Inoculation (I)	2	5.61	0.0001	93.58	0.0001
S * I	10	9.68	0.0001	44.71	0.0001
Error	3480	0.28		5.09	

Table 3. Summary Analysis of Variance on the Effect of *Pisolithus tinctorius* Inoculation on Caliper and Height Growth of six pine species, analyzed by Species.

			Caliper Growth		Height Growth	
	Source	df	MS	Pr > F	MS	Pr > F
P. aristata	Inoculation	2	2.73	0.0001	667.34	0.0001
	Error	569	0.18		2.35	
P. edulis	Inoculation	2	0.11	0.4712	130.33	0.0001
	Error	582	0.14		2.89	
P. nigra	Inoculation	2	2.89	0.0001	24.39	0.0002
	Error	583	0.28		2.86	
P. ponderosa	Inoculation	2	20.56	0.0001	135.86	0.0001
	Error	585	0.46		3.72	
P. strobiformis	Inoculation	2	6.25	0.0001	42.29	0.0228
	Error	583	0.25		11.12	
P. sylvestris	Inoculation	2	21.43	0.0001	613.52	0.0001
	Error	578	0.37		7.56	

Table 4. Analysis of Variance Table for the Effect of *Pisolithus tinctorius* Inoculation and Growth Media Composition on *Pinus ponderosa* Shoot Caliper and Height Growth After 20 Weeks.

		Caliper Growth		Height Growth	
Source	df	MS	Pr > F	MS	Pr > F
Model	8	20.35	0.0001	116.22	0.0001
Media (M)	2	14.83	0.0001	39.81	0.0001
Inoculation (I)	2	3.52	0.0004	150.08	0.0001
M * I	4	31.52	0.0001	137.55	0.0001
Error	1744	0.44		3.36	

Table 5. Influence of *Pisolithus tinctorius* Inoculation Treatment and Growth Media Composition on Caliper and Shoot Height Growth of Container Grown *P. ponderosa* Seedlings. **(should inoculation say 10 weeks?)**

Media Composition	Inoculation	Caliper (mm) (mean ± S.E.)	Shoot Height (cm) (mean ± S.E.)
Light (121)	None	2.5 ± 0.05	7.6 ± 0.13
Light (121)	@ 6 weeks	3.0 ± 0.05	7.6 ± 0.13
Light (121)	@ 12 weeks	3.5 ± 0.05	8.6 ± 0.13
Medium (111)	None	3.0 ± 0.05	8.4 ± 0.13
Medium (111)	@ 6 weeks	2.9 ± 0.05	6.4 ± 0.13
Medium (111)	@ 12 weeks	3.2 ± 0.05	8.1 ± 0.13
Heavy (211)	None	3.6 ± 0.05	9.0 ± 0.13
Heavy (211)	@ 6 weeks	3.3 ± 0.05	8.0 ± 0.13
Heavy (211)	@ 12 weeks	3.0 ± 0.05	7.4 ± 0.13

Table 6. Categorical Analysis of Variance Table for First Year Survival of Seedlings of Seven Pine Species Receiving Differing *Pisolithus tinctorius* Inoculations.

Source	df	Chi-Square	Observed Significance Level
Intercept	1	51.78	0.0001
Species	6	47.46	0.0001
Inoculation	2	5.12	0.0774
Block	7	198.83	0.0001
Species * Inoculation	12	32.77	0.0011
Species * Block	42	124.16	0.0001
Inoculation* Block	14	11.02	0.6843
Species*Inocul.*block	84	186.30	0.0001

Table 7. Summary Categorical Analysis of Variance Table for the Effect of *Pisolithus tinctorius* Inoculation on First-Year Survival of Seven Pine Species by Species.

	Source	df	Chi-Square	Observed Significance Level
P. aristata	Inoculation	2	7.0	0.0300
	Block	7	48.9	0.0001
	Inoculation * Block	14	26.0	0.0300
P. edulis	Inoculation	2	0.8	0.6800
	Block	7	48.7	0.0001
	Inoculation * Block	14	29.6	0.0100
P. flexilis	Inoculation	2	1.6	0.4500
	Block	7	64.8	0.0001
	Inoculation * Block	14	22.5	0.0700
P. nigra	Inoculation	2	17.2	0.0001
	Block	7	35.7	0.0001
	Inoculation * Block	14	30.8	0.0100
P. ponderosa	Inoculation	2	4.3	0.1200
	Block	7	34.4	0.0001
	Inoculation * Block	14	20.2	0.1200
P. strobiformis	Inoculation	2	6.7	0.0300
	Block	7	18.8	0.0100
	Inoculation * Block	14	39.9	0.0001
P. sylvestris	Inoculation	2	0.9	0.6500
	Block	7	38.4	0.0001
	Inoculation * Block	14	32.5	0.0001