Use of low nitrogen fertilizer as a strategy for maintaining mycorrhizal colonization on whitebark pine seedlings inoculated with native fungi in the greenhouse

Erin R Lonergan and Cathy L Cripps

Whitebark pine seedling. Photo by Cathy Cripps

CONVERSIONS
(°C x 1.8) 1 32 = °F
1 m = 3.3 ft
1 cm = 0.4 in
1 ml = 0.034 oz

ABSTRACT

Inoculation of whitebark pine (*Pinus albicaulis* Engelm. [Pinaceae]) with native ectomycorrhizal fungus *Suillus sibiricus* (Bonard.) Singer was investigated under a variety of nursery scenarios. Because fertilization often prevents mycorrhizal colonization in the greenhouse, we tested a low nitrogen (N) fertilizer. In general, ectomycorrhizal abundance was greater in longer containers (21 cm compared with 14 cm); when inoculum (slurry) originated from fresh, rather than dried, sporocarps; and when seedlings were subsequently given very low (13 ppm N every other week) or no additional N fertilizer. Slurry type interacted with container length, and fertilizer rate and colonization rates were all low on seedlings in short containers, except for those that were not fertilized and given slurry from dried sporocarps. Results show that drying and storing sporocarps for future use is possible in slurry although certain conditions might apply. No differences were observed in colonization for the inoculation methods tested, and injection is recommended over the drip method for ease of application. Further research is necessary to refine and optimize fertilizer regime and container type and size for whitebark pine seedlings to be inoculated in the greenhouse prior to outplanting on high-elevation restoration sites. Whitebark pine is currently awaiting official listing as an endangered species in the US and is already listed as such in Canada.

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KEY WORDS
fertilization, native ectomycorrhizal fungi, mycorrhiza, nursery, *Pinus albicaulis*, restoration, *Suillus*

NOMENCLATURE
Fungi: Index Fungorum (2012)
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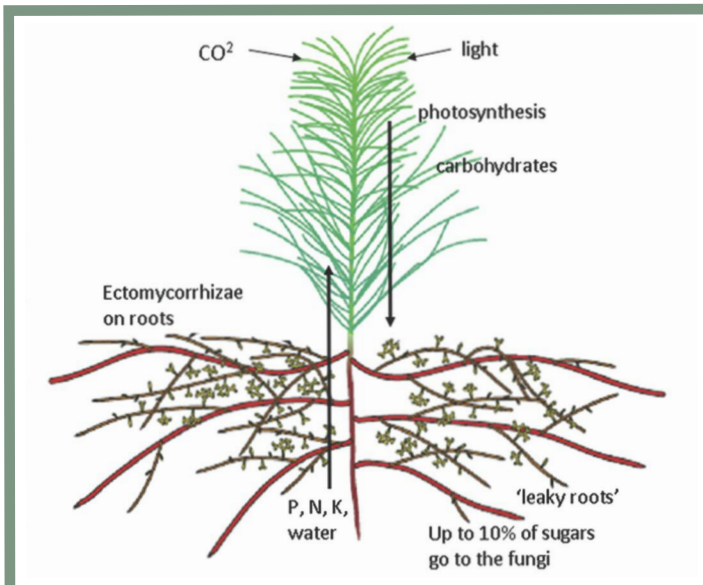
Whitebark pine (*Pinus albicaulis* Engelm. [Pinaceae]) (WBP) is a 5-needle stone pine that produces extensive forests at tree line in western North America (Ellison and others 2005; Lantz 2010). WBP forests have been reduced to a fraction of their former range primarily by white pine blister rust (*Cronartium ribicola* J.C. Fisch.) and mountain pine beetles (*Dendroctonus ponderosae* Hopkins [Coleoptera: Curculionidae]) (Burr and others 2001; Mahalovitch and Dickerson 2004; Schwandt 2006; Keane and Parsons 2010; Logan and others 2010; Tomback and Achuff 2010). Large reforestation efforts are ongoing and WBP is being grown in container nurseries for this purpose (Schwandt 2006; Keane and others 2012), but survival rates of outplanted seedlings are low (Izlar 2007).

One tool that has been used with other pine species to enhance outplanting success is inoculation of seedlings with ectomycorrhizal fungi in the greenhouse; however, results vary depending on the host plant, fungus used, soil type, and site conditions (Wiensczyk and others 2002; Steinfeld and others 2003; Quoreshi and others 2009; Lehto and Zwiazek 2011). Mycorrhizal inoculation can also potentially reduce fertilizer, irrigation, and pesticide expenses and protect against root pathogens in the nursery (Whipps 2004). This strategy has not been employed in WBP production, and a commercial inoculum specific to 5-needle pines is not available. Commercial products are not currently recommended for sensitive whitebark pine systems as they could introduce alien fungi, or fungi that form mycorrhizae with competing tree species (Cripps and Grimme 2011; Keane and others 2012).

Sixty years ago the Federal Forest Nursery in Austria began inoculating European stone pine seedlings (*Pinus cembra* L.) with high-elevation adapted native fungi for reforestation (Moser 1956; Heumader 1992). Inoculation, along with improved silviculture techniques, greatly enhanced seedling survival in the field (Weisleitner 2008). The main ectomycorrhizal fungi used with success in Austria are several species of *Suillus* (Moser 1956; Schmid 2006). Some of these same or related species have recently been found with WBP in the Rocky Mountains (Moser 2004; Cripps and others 2008; Mohatt and others 2008; Cripps and Antibus 2011). These suilloid fungi are specific for 5-needle pines (Bruns and others 2002) and could confer advantages to the pines and not to competing tree species such as subalpine fir (*Abies lasiocarpa* (Hook.) Nutt. [Pinaceae]). Screening trials of native ectomycorrhizal fungi from WBP forests in the Rocky Mountains found that one of these, *Suillus sibiricus* (Bonord.) Singer, outperformed other ectomycorrhizal fungi in the greenhouse (Cripps and Grimme 2011). *Rhizopogon* species are commonly used in commercial inoculum in US nurseries (Amaranthus 2002), but the native species of *Rhizopogon* tested with WBP lagged significantly behind *Suillus* species in mycorrhizal formation in the greenhouse (Cripps and Grimme 2011).

Inoculating seedlings during nursery operations can be challenging because of interactions with fertilizers, substrates, watering regimes, and pest management procedures. Standard protocols for growing high-quality seedlings are not often conducive to colonization by native ectomycorrhizal fungi in the greenhouse (Castellano and others 1985; Castellano and Molina 1989; Khasa and others 2001; Rincon and others 2005). In small-scale early trials, we found that typical fertilizer regimes inhibited the formation of ectomycorrhizae on WBP seedlings (Cripps and Grimme 2011). Studies using other conifers, however, have shown that modified fertilizer regimes can be conducive to ectomycorrhizal colonization (Gagnon and others 1988; Quoreshi and Timmer 1998; Khasa and others 2001). Exponential fertilization has been tried on the 5-



Ectomycorrhizae are structures formed of fungal tissue covering short roots of plants. Carbohydrates from the plant are transferred to the fungus, which surrounds the outer root cells. Fungal hyphae extend into the soil and enhance nitrogen uptake into the plant.

Figure from Brundrett MC. 2008. Ectomycorrhizas. In: *Mycorrhizal Associations: The Web Resource*. Version 2.0. 7 Sept 2012 (mycorrhizas.info), amended by C. Cripps

needle *Pinus monticola* Rydb., but not in conjunction with mycorrhizal inoculation (Dumroese and others 2005). In addition, inoculum type and amount, method of application, and container style need to be explored to optimize the timely formation of ectomycorrhizae under greenhouse conditions (Landis and others 1989b; Khasa and others 2009; Quoreshi and others 2009; Repáč 2011).

Our main objective was to determine if WBP seedlings inoculated with native ectomycorrhizal fungi could maintain this mutualism when low nitrogen (N) fertilizer is subsequently applied in the greenhouse. We hypothesized that a low N fertilizer would be less likely to interfere with the established mutualism since *S. sibiricus* (and other suilloid fungi) are known to be involved primarily with enhancing N uptake in plants (Keller 1996; Cripps and Antibus 2011). This method is analogous to the use of a low phosphorous fertilizer for arbuscular fungi that has been shown to allow mycorrhizal colonization in the greenhouse; this strategy ultimately enhanced survival of several plant species in field trials (Meikle and Amaranthus 2008). If successful with WBP, this inoculation would allow fertilization to continue before outplanting, thus enhancing the stature and health of seedlings by 2 strategies, fertilization and mycorrhizal inoculation. This approach has application for other container-grown conifers as well.

In addition, 3 other variables were examined for optimizing mycorrhizal formation on WBP seedlings in the greenhouse: 2 types of spore slurries (fresh and dried), 2 methods of inoculation (injection or drip), and 2 container lengths. Short containers were tested because seedlings grown in long containers are difficult to outplant in the shallow, rocky soils found at the high-elevation WBP restoration sites.

MATERIAL AND METHODS

Seedlings

WBP seedlings grown from seeds originating from Preston Park (lat 48°43′45″N, long 113°39′03″W, 2672 m elevation, Glacier National Park) were sown at the USDA Forest Service nursery in Coeur d'Alene, Idaho, in February 2009. The nursery typically grows WBP in long Ray Leach cone-tainers (3.8 cm diameter × 21 cm depth; Stuewe & Sons, Tangent, Oregon) and was testing a mixture of Canadian Sphagnum peat moss and composted bark (7:3, v:v) at this time. At the Coeur d'Alene Nursery, seedlings are fertilized every 8 to 12 d with a $20N:7P_2O_5:10K_2O$ fertilizer (Peter's Professional, The Scotts Company, Marysville, Ohio) with STEM (soluble trace element mix, The Scotts Company,) micronutrients (Eggleston 2010) followed by a $4N:25P_2O_5:15K_2O$ finisher. In April 2010, 500 seedlings from this crop were transferred to the Plant Growth Center (PGC, Montana State University [MSU], Bozeman, Montana) where fertilization was stopped to allow conditions for mycorrhization to develop. Seedlings were then grown under standard greenhouse conditions (22 °C day and 18 °C night temperatures, 16 h photoperiod). Approximately half ($n = 254$) of the WBP seedlings were transplanted to short Ray Leach cone-tainers (3.8 cm × 14 cm, 115 ml) and the other half ($n = 260$) returned to their original long Ray Leach cone-tainers (3.8 cm × 21 cm, 164 ml) from the Coeur d'Alene Nursery (Figure 1). Roots of seedlings being transplanted into short containers were first trimmed with scissors. All containers were filled with MSU Soil Mix 3, a mixture of pasteurized MSU mix, vermiculite, and sifted Canadian Sphagnum peat moss (2:2:1 by volume) with an average pH of 5.66. The pasteurized MSU components (1:1:1 by volume) are loam, peat moss, washed concrete sand, and AquaGro 2000 G wetting agent blended in at a rate of 0.59 kg/m^3 (1 lb/yd^3) of soil mix.

Spore Slurry Types and Inoculation Methods

Sporocarps (mushrooms) of *Suillus sibiricus* were collected in WBP forests in Montana in September 2010. Two large collections (Gallatin, 2800 m; Flathead County, 1000 m) were used to create slurries based on sporocarp condition: fresh and dried (Figure 1). Half of the sporocarps were immediately cleaned and the hymenium (pore surface) removed and cut into small pieces. These pieces were placed in a coffee grinder with 10 ml of sterile distilled water and ground for approximately 1 min and then strained into 400 ml of water. The spore content of the slurry was estimated using a hemacytometer and further diluted with distilled water to a spore count of approximately 1×10^6 spores/ml. The resulting fresh slurry was refrigerated for 1 mo in glass bottles. The remaining half of the sporocarps were immediately dehydrated on a drier and stored in plastic bags for about 1 mo. Just prior to inoculation these sporocarps were made into what is subsequently termed "dried slurry" using the same method as used for fresh sporocarps.

In November 2010, the 21-mo-old WBP seedlings from both container sizes were inoculated with either the fresh or dried slurry using either a drip or an injection method. For the drip method, seedlings were removed from containers, 5 ml of spore slurry was dripped onto the exposed roots using a glass pipette, and seedlings were returned to their containers. For the injection method, 5 ml of spore slurry was injected directly onto the growing medium using an Allflex 50 ml repeat syringe (Figure 1). Both methods delivered approximately 5×10^6 spores to each seedling.

Fertilization Regimes

Seedlings were vernalized in a cold room (approximately 4 °C) from January 2011 through March 2011 to mimic natural conditions. In April, seedlings were moved to the PGC (at MSU) and the fertilization regimes (high, low, and control) were started and continued for 22 wk (Figure 2). The fertilizer was Phosgard $4N:25P_2O_5:15K_2O$ liquid NPK fertilizer (JH Biotech Inc, Ventura, California). All seedlings were fertilized

Figure 1. Dried and fresh sporocarps of *Suillus sibiricus* for spore slurries; Allflex 50-ml repeat syringe for the injection of spore slurries into the soil (inject method); glass pipette used for dripping spore slurries over roots (drip method); and short and long Ray Leach cone-tainers. Photos by Erin Lonergan

from the same solution (13 ppm N); seedlings in the high, low, and control treatments were fertilized once each week, every other week, and never, respectively. Seedlings were checked 3 times per wk and watered to field capacity as needed.

Assessment of Mycorrhizal Colonization

WBP seedlings were assessed by nondestructive techniques for mycorrhizal colonization 3 times throughout the experiment (Figure 2). The January and March 2011 assessments were used to determine if inoculum was viable and if suilloid ectomycorrhizal colonization was occurring prior to initiating the fertilizer treatments. Approximately half of the seedlings were randomly selected and evaluated in January, whereas all seedlings were evaluated in March. The third assessment was completed in November 2011. At the third assessment, all seedlings were evaluated for both frequency (percentage of seedlings with suilloid ectomycorrhizae) and average abundance of ectomycorrhizal colonization (percentage of roots covered). To assess abundance, roots of each seedling were immersed in distilled water and soil particles were removed by gentle agitation. Roots were observed under a dissecting scope to determine percentage of colonization of suilloid ectomycorrhizae, which are recognized by characteristics typical of

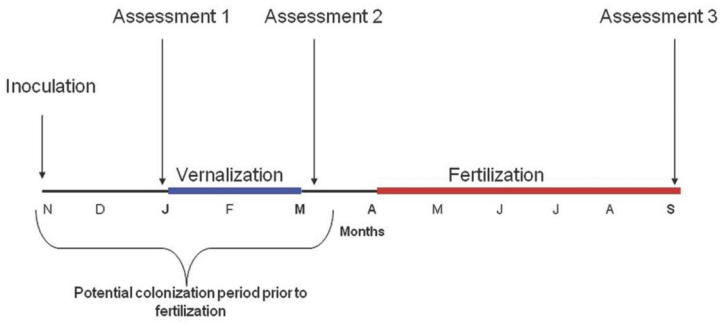


Figure 2. Order and timing of inoculation, vernalization, and fertilization of whitebark pine seedlings. Assessments 1, 2, and 3 are for frequency and Assessment 3 is for abundance of mycorrhizal colonization on roots. Inoculation was in November 2010, Assessment 1 in January 2011, 2 in March 2011, and 3 in November 2011.

S. sibiricus (Figure 3), in particular, the presence of a white plectenchymatous mantle, a coralloid branching pattern, weak rhizomorphs, and a lack of clamp connections (Treu 1990). Periodic checks under the compound microscope helped to confirm identification.

Experimental Design and Data Analysis

The experimental design was for 2 container types (long and short) × 2 slurry types (fresh and dried) × 2 inoculation techniques (drip and injection) × 3 fertilizer rates (high, low, none) (Table 1). The number of seedlings in each combination of treatments varied as we were limited by the number of WBP seedlings available from the Coeur d'Alene Nursery. Because the main goal of this project was to test for an alternative to current fertilization regimes, most seedlings were placed in the fertilized groups (Table 1). In addition, a few seedlings died randomly across treatments, which contributed to a variable number of seedlings assessed. To keep N numbers high with the limited number of seedlings available, there was no replication. WBP seedlings were placed in container trays, and individual trays were rotated each month to compensate for variation in light and other greenhouse conditions.

The 3 assessments of frequency were primarily to determine if dried and fresh slurries were viable under experimental conditions; therefore, frequency was not analyzed statistically. The final, mean percentage of ectomycorrhizal colonization (abundance) of inoculated WBP seedlings was analyzed using a four-way factorial analysis of variance (ANOVA) in SPSS (IBM Corporation 2011). The analysis included 4 main effects (container length, slurry type, inoculation method, fertilization level) and all possible interactions. Levine's test of equal variance and box plot analysis indicated that the assumption of equal variance was not met and data were transformed (square root) prior to analysis. Any statistical differences detected among treatment combinations were analyzed further using pairwise comparisons. Means for each fertilizer treatment were separated by Tukey's honest significance difference (HSD) test ($\alpha = 0.05$).

Figure 3. Suillus sibiricus ectomycorrhizae on whitebark pine seedlings roots in the greenhouse. White clustered "hand-like" ectomycorrhizae are characteristic. Photo by Cathy Cripps

TABLE 1

Experimental setup and number of seedlings used in each treatment.

Container length	Slurry type	Inoculation method	High fertilizer	Low fertilizer	No fertilizer
Long	Fresh	Drip	18	17	5
		Inject	21	16	8
	Dried	Drip	13	14	7
		Inject	18	18	8
Short	Fresh	Drip	12	18	5
		Inject	22	19	9
	Dried	Drip	9	15	6
		Inject	16	13	6

RESULTS

Frequency of Mycorrhizal Colonization: Assessments 1, 2, and 3

Three mo after inoculation (Assessment 1 in January), 91% of WBP seedlings inoculated with slurry made from fresh sporocarps showed signs of ectomycorrhizal colonization whereas colonization occurred for 29% of seedlings inoculated with slurry made from dried sporocarps (Figure 4). Five mo after inoculation and following vernalization (Assessment 2 in March), 80% of seedlings inoculated with fresh slurry showed signs of ectomycorrhizal colonization and 34% of seedlings inoculated with dried slurry had ectomycorrhizae (Figure 4).

At the end of the fertilizer treatment (Assessment 3 in September), 97% of the seedlings inoculated with fresh slurry and 92% of those inoculated with dried slurry had ectomycorrhizae (Figure 4). Thus, a high percentage of the seedlings had suilloid ectomycorrhizae at the end of the experiment, and frequency ranged between 90 to 100% for all groups.

Abundance of Ectomycorrhizal Colonization

At the end of the growth portion of the experiment, the root system of each WBP seedling was examined to determine the percentage covered by ectomycorrhizae (abundance). Mycorrhizal colonization was influenced by fertilizer treatment, container length, fertilizer × slurry type interaction, container length × slurry type interaction, and fertilizer × container length × slurry type interaction (Tables 2 and 3; Figure 5). The significant fertilizer × slurry type interaction ($P = 0.049$, $f_{2, 289} = 3.05$) shows that mycorrhizal colonization was higher on fertilized seedlings (low and high treatments) when fresh slurry was added, but this was not true for unfertilized seedlings. The interaction between container length × slurry type ($P = 0.006$, $f_{1, 289} = 7.64$) revealed that seedlings in long containers were better colonized when fresh slurry was applied; however, this was not true in all cases for seedlings in short containers. The three-way interaction between fertilizer × container × slurry type ($P = 0.002$, $f_{2, 289} = 6.19$) showed that fertilized seedlings had the highest colonization rate when planted in long containers with fresh slurry added. Unfertilized seedlings in long containers also had higher colonization with fresh slurry but unfertilized seedlings in short containers produced more mycorrhizae with dried slurry.

TABLE 2

Four-way ANOVA for effects of fertilizer treatment, container length, slurry type, and inoculation method on the abundance of ectomycorrhizal colonization.

Source	Type III SS	df	F-stat	Significance
Intercept	**7264.756**	**1**	**1696.21**	**0.854**
Fertilizer group (F)	**172.613**	**2**	**20.151**	**0.000****
Container length (C)	**100.406**	**1**	**23.443**	**0.000****
Slurry type (S)	**11.795**	**1**	**2.754**	**0.098**
Inoculation method (I)	11.655	1	2.721	0.100
F x S	26.131	2	3.051	0.049*
F x C	4.766	2	0.556	0.574
F x I	1.557	2	0.182	0.834
C x S	32.738	1	7.644	0.006*
C x I	0.135	1	0.032	0.859
S x I	0.011	1	0.002	0.960
F x C x S	53.031	2	6.191	0.002*
F x C x I	7.195	2	0.840	0.433
F x S x I	15.440	2	1.802	0.167
C x S x I	1.111	1	0.260	0.611
F x C x S x I	6.027	2	0.704	0.496

Notes: Whitebark pine seedlings inoculated with the native fungus *Suillus sibiricus* in the greenhouse. Data square root transformed. Main effects are in bold.

Significant at * P < 0.05 and **P < 0.001.

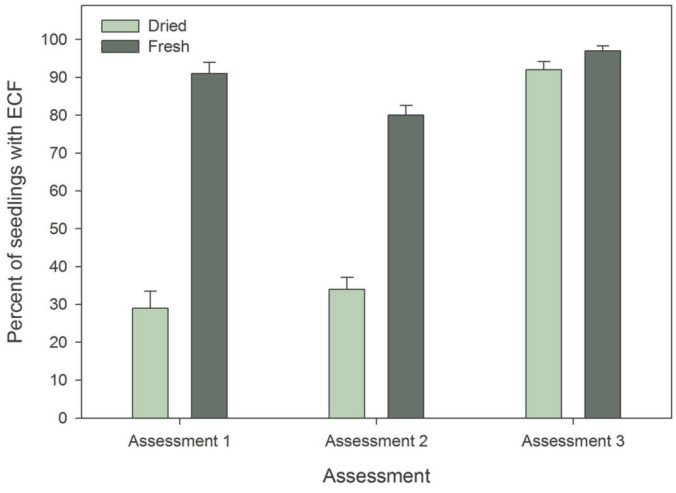


Figure 4. Effects of spore slurry type on *frequency* (%) of ectomycorrhizal colonization on whitebark pine seedlings for Assessments 1, 2, and 3 with 95% confidence bars. Spore slurries were made from either fresh or dried sporocarps. Assessment 1 was in January prior to vernalization, Assessment 2 was in March after vernalization, and Assessment 3 was in November after fertilization treatments were applied.

Effect of Fertilizer Treatment

Ectomycorrhizae were maintained to some degree on seedlings fertilized with all fertilizer regimes, which was a main goal of the experiment. The abundance of ectomycorrhizal colonization, however, differed among fertilizer treatments averaged across container length, slurry type, and inoculation method ($P = <0.001$, $f_{2,2\ 89} = 20.15$) (Table 2). With one exception, ectomycorrhizal colonization was highest on unfertilized seedlings (68%, 46%, 54%), reduced with light fertilization (43%, 34%, 21%), and further reduced with heavier fertilization (34%, 23%, 17%), for long-fresh, long-dried, short-dried treatments, respectively (Figure 5). Fertilization treatment had no effect on the colonization of seedlings in short containers given fresh slurry ($P = 0.33$, $f_{2, 289} = 1.12$); here colonization rates were all low (21%, 28%, 22%).

Effect of Container Length

A significant effect occurred for container length ($P = < 0.001$, $f_{1, 289} = 23.44$) (Table 2). In general, ectomycorrhizal colonization was higher on WBP seedlings in long containers

TABLE 3

Effects of fertilizer treatment, container length, and slurry type on the mean abundance of ectomycorrhizal colonization.

Fertilizer treatment	Container length	Slurry type	Mean (%)	Standard error
High	Long	Dried	23.26	3.71
		Fresh	34.92	3.30
	Short	Dried	16.08	4.13
		Fresh	22.65	3.54
Low	Long	Dried	35.31	3.65
		Fresh	43.03	3.59
	Short	Dried	21.25	3.90
		Fresh	28.54	3.39
None	Long	Dried	46.00	5.33
		Fresh	66.15	5.72
	Short	Dried	54.17	5.96
		Fresh	22.86	5.51

Notes: Whitebark pine seedlings inoculated with the native fungus *Suillus sibiricus* in the greenhouse. Data pooled for inoculation method since there were no significant effects.

with one exception; that is, unfertilized seedlings in short containers given dried slurry also had high colonization (Figure 5). However, seedlings in long containers had the highest colonization when they were not fertilized, and colonization declined as fertilizer rate increased, regardless of slurry type. Differences among fertilizer treatments were significant for seedlings in long containers, whether they were inoculated with fresh ($P < 0.001$, $f_{2, 289} = 12.43$) or dried sporocarps ($P = 0.002$, $f_{2, 289} = 6.39$).

Seedlings in short containers were less well colonized by ectomycorrhizal fungi for all treatment combinations in comparison with those in long containers, again with the exception of unfertilized seedlings given dried slurry (Figure 5). These unfertilized seedlings had significantly higher colonization than unfertilized seedlings given fresh slurry ($P < 0.001$, $f_{2, 289} = 13.20$). Again, no differences occurred in ectomycorrhizal colonization among seedlings in short containers for the group inoculated with fresh slurry.

Effect of Slurry Type

When fresh slurry was applied, WBP seedlings in long containers were better colonized for each of the 3 fertilizer treatments in comparison with those given dried slurry ($P = 0.02$, $f_{1, 289} = 5.12$; $P = 0.07$, $f_{1, 289} = 3.14$; $P = 0.006$, $f_{1, 289} = 7.77$; for high, low, and none treatments, respectively). Fresh slurry also produced slightly higher colonization on fertilized seedlings in short containers but differences were not significant, while the use of dried inoculum for unfertilized seedlings in short containers resulted in higher colonization ($P < .0001$, $f_{2, 289} = 14.40$) (Figure 5).

Effect of Inoculation Method

The analysis of inoculation methods (drip or injection) showed no statistical differences at the $P = 0.05$ level, and there were no significant interactions for this variable (Table 2). Therefore, data for the variable of inoculation method are pooled in Figure 5 and Table 3.

DISCUSSION

Numerous studies have shown that fertilization, especially with N, can reduce or eliminate ectomycorrhizal colonization of inoculated conifers in the greenhouse (Castellano and others 1985; Gagnon and others 1988; Arnebrant 1994; Smith and

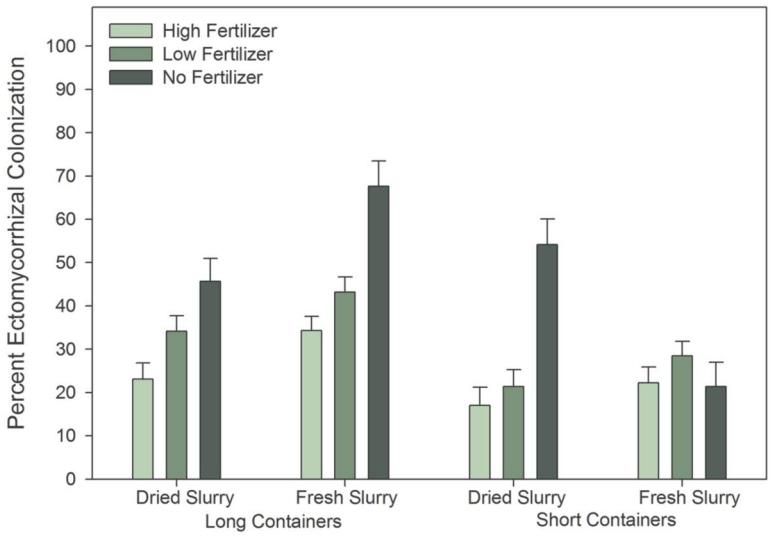


Figure 5. Mean percentage of abundance of ectomycorrhizal colonization of whitebark pine seedlings inoculated with *Suillus sibiricus* as a function of fertilizer group, container length, and slurry type with 95% confidence bars. Data from inoculation method are pooled since no significant difference was observed.

Read 2008). This finding is also true for WBP, for which weekly applications of 200 ppm of N fertilizer (Peters General Purpose $20N:20P_2O_5:20K_2O$, The Scotts Company) resulted in negligible mycorrhizal colonization on seedling roots (Cripps and Grimme 2011). The low N fertilizer (Phosgard $4N:25P_2O_5:15K_2O$) used here did allow ectomycorrhizal colonization to be maintained on WBP seedlings across treatments. Frequency estimates at the end of the experiment showed that most seedlings (more than 90%) had evidence of mycorrhizae on their roots. The amount of colonization (abundance), however, was reduced with fertilization every other week, and reduced even further with application once a week in comparison with unfertilized controls (at least for seedlings in long containers and those in short containers given dried slurry). The percentage of well-colonized roots is comparable with other greenhouse inoculations where seedling roots are not completely colonized, possibly due to differences in container conditions or genetic variation in seedlings (Brundrett and others 2005). Low levels of ectomycorrhizal colonization are not functionally sufficient in some cases (Marx and Cordell 1988), although the minimum needed to enhance WBP survival in the field is not known.

Nonetheless, fertilization is necessary for the production of container-grown pines (Landis and others 1989a), and all WBP seedlings used in restoration are currently grown in containers. High fertilization levels can promote nursery fungi such as *Thelephora* Pers., E-strain, and root pathogens (Quoreshi and others 2009); WBP seedlings can host a variety of fungal pathogens in the greenhouse (James and Burr 2000; Dumroese 2008). The increase in substrate pH with fertilization can be inhibitory to many native ectomycorrhizal fungi and particularly for spore germination (Castellano 1996; Rincón and others 2005).

High N in particular can have detrimental effects on ectomycorrhizal formation on roots (Wallender and Nylund 1991; Brunner and Brodbeck 2001). In the greenhouse, high N fertilizers have been shown to reduce ectomycorrhizal colonization on *Pinus contorta* Douglas ex Loudon (Ekwebelam and Reid 1983), *Pinus halepensis* Mill. (Diaz and others 2010), and *Picea mariana* (Mill.) DuRoi (Gagnon and others 1988) seedlings, to give a few examples. Many of the native fungi used in inoculations, including those used on WBP, are known to be involved with N acquisition, so results could be more applicable to these ectomycorrhizal fungi (Keller 1996).

Exponential fertilization could be more conducive to mycorrhizal colonization in the greenhouse and is achieved by progressively increasing nutrient application to correspond with seedling growth rates. Ectomycorrhizal colonization of *Picea mariana* was increased significantly in the greenhouse with exponential fertilization in comparison with conventional fertilization (Quoreshi and Timmer 1998). For the 5-needle western white pine (*Pinus monticola* Rybd.), a lower nutrient application rate achieved through exponential fertilization reduced overall fertilization by 45% compared with conventionally fertilized seedlings (Dumroese and others 2005). These seedlings were not inoculated in the greenhouse, but the exponentially fertilized seedlings had higher ectomycorrhizal colonization the year following outplanting.

Nitrogen loading before outplanting can increase survival of container-grown conifers under some circumstances (van den Driessche 1987; Timmer 1996; Rikala and others 2004). A more recent study reported that *Pinus palustris* Mill. seedlings grew larger with added N, but survival was unaffected by the rate of N addition, at least above a certain threshold (Jackson and others 2012). Foliar N also can be enhanced by ectomycorrhizal fungi depending on the conifer species and fungal isolates involved (Gagnon and others 1988; Chakravarty and Chataropaul 1989; Heumader 1992; Quoreshi and Timmer 1998; Amaranthus and others 2005; Rincon and others 2005). For example, significant increases in N uptake have been reported for inoculated seedlings of *Pinus halepensis* (Rincon and others 2007) and *Picea mariana* (Gagnon and others 1988; Quoreshi and Timmer 1998) in comparison with non-inoculated controls. More applicably, European stone pine seedlings associated with *Suillus placidus* (Bonord.) Singer in the nursery had significantly higher foliar N compared with non-inoculated seedlings (Heumader 1992). Green manure is used to maintain this mycorrhizal association in lieu of chemical fertilizer, and seedlings are planted in biodegradable pots (Heumader 1992). In the experiment reported here, foliar N was not assessed.

Ectomycorrhizal inoculation of nursery-grown conifers has been shown to improve seedling survival in the field on sites where natural inoculum is lacking (Wiensczyk and others 2002; Steinfeld and others 2003; Parladé and others 2004; Gagne and others 2006; Menkis and others 2007). In areas where native ectomycorrhizal fungi are present, nursery inoculation can benefit seedling survival by enhancing access to nutrients and water during the critical establishment period (Ortega and others 2004; Quoreshi and others 2009; Lehto and Zwiazek 2011). For example, survival of *Pinus ponderosa* P. Lawson & C. Lawson seedlings planted in harsh, dry areas increased 56% on one site and 30% on another when seedlings were inoculated with *Rhizopogon* species (Steinfeld and others 2003). Similarly, 3.5 y after outplanting, survival of *Pinus pinea* L. seedlings was 20% higher for seedlings inoculated with *Rhizopogon roseolus* (Corda) Th. Fr., and the increase was enough to justify inoculation expenses (Parladé and others 2004). Survival of European stone pine seedlings in Austria increased from 50 to 90% after inoculation with native ectomycorrhizal fungi in combination with intensive silviculture techniques, and this method is maintained today (Moser 1956; Schmid 2006).

The ectomycorrhizal fungi used in seedling inoculation do not always persist after outplanting; the use of appropriate native fungi in the greenhouse may circumvent the need for fungal replacement in the field. In Austria, 50 y after inoculation of European stone pine seedlings with 3 indigenous species of *Suillus,* including *S. sibiricus,* molecular techniques identified all 3 original *Suillus* species as still present and colonizing root systems (Schmid 2006).

Container size can play an important role in shaping the morphology and physiology of seedling root systems. Long, narrow containers are typically recommended for growing native plants for restoration to reduce the effects of limiting soil moisture (Landis and others 1990; Dominguez-Lerena and others 2006; Landis and others 2010). Yet, planting WBP seedlings with long root systems in the shallow, rocky soils often associated with high-elevations sites has been difficult to accomplish. In general, our data show that seedlings in short containers had fewer mycorrhizae than those in long containers, but additional exploration of alternative container types and sizes for nursery-grown seedlings would be valuable (Landis and others 2010). Containers of various sizes tested with ponderosa pine seedlings showed that container specifications can make a difference in growth on outplanting and should be selected for particular site conditions (Pinto and others 2011). In this study, seedlings planted in shorter containers (10.3 cm depth) outperformed those in longer containers (22.7 cm) on a xeric site, possibly because correctly planting seedlings in shorter containers is easier than in long ones (Pinto and others 2011). In one study, roots of WBP seedlings retained their long container shape 5 y after outplanting and did not grow out into native soil (Trusty and Cripps 2011). Thus, mycorrhization in the field may also be inhibited by container type.

Successful inoculation of containerized nursery-grown seedlings has been achieved through the use of a variety of inoculum types (Castellano and others 1985; Boyle and Robertson 1987; Repáč 1996a, 1996b, 2007, 2011). In a large experiment, spore slurries were found to be more effective, less costly, and more efficient than mycelial suspensions overall (Brundrett and others 2005), and this was shown to be true for *Suillus* inoculum as well (Rincon and others 2007). For WBP, spore slurries made from fresh sporocarps were more effective than mycelial inoculum under greenhouse conditions in small trials (Cripps and Grimme 2011). The viability of suilloid spore slurries has been maintained with refrigeration up to 3 y (Castellano and Molina 1989), but viability has also been shown to decline in 3 mo depending on storage conditions (Torres and Honrubia 1994). Declines can reflect dormancy rather than death, and results may depend on the assessment method.

In this WBP experiment, the use of dried sporocarps in slurry was tested as a way to alleviate the problem of the time gap between sporocarp appearance (October) and the inoculation time frame that occurs months later. Dried sporocarps may also remedy the difficulty of locating fruiting bodies in drought-prone sites every year. If sporocarps could be dried and stored for a time before use, shelf life would be essentially extended. While not as many seedlings showed signs of mycorrhizal colonization in the first 2 frequency assessments, at the completion of the experiment, no practical difference was observed in the frequency of suilloid mycorrhizae between dried and fresh inoculum. A latency period brought on by drying of the sporocarps or dormancy factors associated with suilloid spores could explain the lower colonization early on; perhaps dried spores need more time or vernalization to germinate (Aime and Miller 2002). Results here suggest that dried fruiting bodies of *Suillus sibiricus* can be stored for at least 1 mo and subsequently be used in spore slurry inoculation of 5-needle pines, although longer storage times need to be tested.

For each type of fungal inoculum, whether mycelial or spores, an array of effective application methods exists (Repáč 2011). For spore inoculum, the most common inoculation method is the application of spores suspended in water (spore slurry) to the seedling soil substrate through drenching, irrigation, or injection. Inoculation of WBP seedlings was previously successful using an Allflex 50 ml repeat syringe to inject spore slurry into the soil surrounding seedlings (Cripps and Grimme 2011). In the current study, we hypothesized that dripping spore slurry over the whole root system (drip method) might increase mycorrhizal colonization; however, at the end of the experiment no differences were observed between the 2 methods. From a logistical viewpoint, the injection method was more efficient, with the drip method potentially spreading disease due to extra handling of seedlings.

Currently, our data suggest that combining low N fertilization with ectomycorrhizal inoculation of WBP seedlings in the nursery is possible. Further research is necessary to refine and optimize the fertilization regime and container type and size for WBP seedlings slated to be planted on high-elevation sites. For WBP restoration, severe burns, ghost forests, and areas not previously inhabited by whitebark pine that may lack native inoculum are sites that could potentially benefit from greenhouse inoculation with native ectomycorrhizal fungi.

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AUTHOR INFORMATION

Erin R Lonergan
Research Associate
lerin3@montana.edu

Cathy L Cripps
Associate Professor
ccripps@montana.edu

Plant Sciences and Plant Pathology Department
Montana State University
Bozeman, MT 59717