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Arbuscular mycorrhizal inoculation following biocide treatment improves Calocedrus decurrens survival and growth in nursery and outplanting sites

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Abstract

Commercial production of tree seedlings often includes various biocidal soil treatments for disease control. Such treatments can be effective in eliminating or reducing disease organisms in the soil, but may also eliminate non-targeted beneficial soil organisms, such as mycorrhizal fungi, that improve seedling performance, both in the nursery as well as the outplanted environment. The arbuscular mycorrhizal fungal (AMF) relationship has been verified for some important western coniferous species such as Calocedrus decurrens (Torr.) Florin (incense cedar), Sequoia sempervirens, (D.Don) Endl

(coastal redwood) and Thuja plicata J. Donne ex D. Don (western red cedar).

This study was designed to determine the response of Calocedrus decurrens after soil fumigation with and without the addition of phosphorous fertilizer and a commercial mycorrhizal inoculant containing Glomus intraradices. Calocedrus seedling performance was monitored in both the nursery and outplanted environments.

At the nursery, non mycorrhizal seedlings had significantly less foliar phosphorous levels and uneven growth even when phosphorous fertilizers were applied. Mycorrhizal inoculation at the nursery significantly improved height growth and improved seedling uniformity on treated plots. Seedlings from the nursery beds were then outplanted on two reforestation sites. Mycorrhizal inoculation at the nursery improved survival and growth of seedlings at the outplanted site.

Introduction

Under natural conditions, most plants live in close beneficial association with soil microorganisms called mycorrhizal fungi. These fungi colonize plant roots and extend the root system into the surrounding soil to form an essential link between plant and soil environment. Mycorrhizal mycelia are extensions of the plant root system and are more effective in nutrient and water absorption than plant roots by themselves. The relationship is mutually beneficial

because the fungus receives essential sugars and other compounds from the plant to fuel its activities and in return it increases plant nutrient and water uptake, increases plant resistance to disease and extends protection against a wide variety of environmental extremes (Harley and Smith, 1983; Allen 1991). All conifer species are known to form and be dependent upon the mycorrhizal relationship in their native habitats.

Commercial production of tree seedlings often includes various biocidal soil treatments for disease control. Such treatments can be effective in eliminating or reducing disease organisms, but may also eliminate non-target beneficial soil organisms such as mycorrhizal fungi (Menge 1982; Trappe *et al.* 1984; Kough *et al.*1985). Research has shown mycorrhizal fungi as critical to the uptake of water and nutrients and seedling survival across a wide range of host and field conditions (Amaranthus and Steinfeld 2003; Steinfeld *et al*. 2003; Miller *et al.* 1998; Jackson *et al.* 1998). However, nursery conditions in which water and nutrients are amply provided, can decrease the need and observed benefits of the mycorrhizal relationship. This is especially true when phosphorous is readily available (Browning and Whitney 1992; Harley 1978). Numerous practitioners, however, have observed stunting and uneven growth of conifers following biocidal treatments even after soil analysis reveals adequate levels of soil fertility. Many of these cases of uneven growth and nutrient deficiencies following biocidal treatment have documented improved growth and nutrition when inoculated with the appropriate mycorrhizal fungus (Bartschi *et al.* 1981, Parke 1982; Parke *et al.* 1983). In these

cases, poor growth of many conifer species despite adequate soil fertilization, may be due to the coarse root systems lacking root hairs which mycorrhizal fungi augment by providing increased surface area and enzymes activity to release immobile soil nutrients such as phosphorous, zinc, copper and others (St. John 1979).

Mycorrhizal fungi can profoundly effect seedling performance in the field by mediating nutrient and water uptake and protecting against environmental extremes in the narrow window for seedling establishment (Harley and Smith 1983; Amaranthus *et al.* 2004; Steinfeld *et al.* 2003). A typical forest site generally contains many mycorrhiza-forming fungal species (Amaranthus *et al.* 1996; but populations can be dramatically reduced or eliminated following site disturbance (Amaranthus and Trappe 1993, Dumroese *et al.* 1998, Perry *et al.* 1987.) Seedlings inoculated at the nursery with the appropriate mycorrhizal fungi *before* outplanting have the ability to more quickly assimilate site resources during the critical period for seedling establishment.

The arbuscular mycorrhizal fungal (AMF) relationship has been verified for some important western coniferous species such as Calocedrus decurrens (Torr.) Florin (incense cedar), Sequoia sempervirens, (coastal redwood) and Thuja plicata (western red cedar). This study was designed to determine the response of Calocedrus decurrens after soil fumigation with and without the addition of phosphorous fertilizer and a commercial mycorrhizal

inoculant. Calocedrus performance was monitored in both the nursery and outplanted environments.

METHODS

Nursery

Uniform nursery beds were fumigated with methyl bromide at the USDA Forest Service J. Herbert Stone Nursery in Central Point, Oregon in February 1991. Four replicate plots of four treatments were installed with 1.0 m buffers separating plots. Four plots (2.0 m length and 1.25 m wide) were randomly assigned one of four treatments before sowing Calocedrus decurrens seed:

MYCO/no P

30,000 propagules of Glomus intraradices were added per plot and no phosphorous fertilizer added

MYCO/P

30,000 propagules of Glomus intraradices were added per plot and phosphorous fertilized at a rate of 200 pounds per acre

No MYCO/no P

No Glomus intraradices and no phosphorous was added to the plot

No MYCO/P

No Glomus intraradices was added to the plot and phosphorous fertilized at a rate of 200 pounds per acre

Mycorrhizal inoculum containing spores and root fragments of Glomus intraradices was produced on an inert clay carrier and added to the treated seed beds 2 weeks after fumigation. Mycorrhizal propagule densities were determined using the sugar centrifugation spore extraction method and clearing and staining of the colonized root fragment techniques. After six months in the nursery beds, seedlings were evaluated for mycorrhizal colonization percent, caliper growth, height and seedbed density.

Outplanting Sites

The outplanting sites were two clearcut sites in the Illinois Valley Ranger District of the Siskiyou National forest of southwest Oregon. The seedlings were planted drainage on south and west facing slopes in the Wood Creek and at a mean elevation of 420 and 480 m respectively. Slope steepness ranged from 25 to 50 percent. The soil consisted of fine-loamy mixed mesic Ultic Haploxeralfs, formed in colluvium derived from metavolcanic parent material of 80 –120 cm depth. Coarse fragments in the surface soil averaged 35%. Annual precipitation averages 210 cm, with more than 90 percent of it falling between mid-September and mid-May.

The areas were clearcut in the winter of 1990, broadcast burned in the fall of1991, and planted with Douglas-fir [Pseudotsuga menziesii

(Mirb.)Bong.] seedlings in the spring 1992. The fall 1991 broadcast burn intensity was severe. All surface litter and duff layers, downed woody material less than 20 cm, and leaves and needles were completely consumed by the fire. Following the burn, bare mineral soil was exposed on 70 to 80 percent of the two clearcut sites.

Naturally reoccurring clumps of pioneering hardwoods—primarily the arbutoid or ectomycorrhizal Arbutus menziesii Pursh, Castanopsis chrysophila (Dougl.) A. DC., Lithocarpus densiflorus (Hook. & Arn.) Rehd. and Quercus kelloggii Newb. and the AMF Rhus diversiloba T. & G.—were widespread across the two clearcuts.

Planting Procedure

In April, 1992, four planting blocks of 10 x 10 m were established at each of the two clearcut test sites. Seedlings were sorted on the landing before outplanting to assure seedlings of similar size would be outplanted for each treatment. Each block was located to be entirely on the same aspect and slope and planted with 16 Calocedrus seedlings from each treatment arrayed in a 4x4 pattern with 0.5 m spacing between seedlings and 1.0 m spacing between treatments.

Plastic netting was placed around seedlings following planting to reduce browsing by deer. The stem diameter, 1 cm above the soil surface, was recorded for each seedling at planting time. Seedling survival, stem diameter and leader growth were measured for all surviving seedlings 14 months following outplanting

Mycorrhizal colonization

Two seedlings per treatment and per replication were randomly selected for mycorrhizal colonization percentage at the time of lifting at the nursery seedlings and 14 months after outplanting on the two clearcut sites. Root systems of all seedlings were removed by excavating soil materials with a tile spade in a trench beyond the width and depth of the existing root system. Root systems were taken to the laboratory for analysis of mycorrhizal colonization. Roots were gently washed free of soil and extraneous material. Arbuscular mycorrhizal colonization was determined by cutting fine root samples into segments that would fit handily in small capsules used for clearing and staining. Roots were cleared in 10% KOH solution, steamed for 72h, rinsed with tap water and transferred to 1% HCL solution for 30 min, then rinsed again with tap water. Cleared samples were transferred into a staining solution of 0.5% trypan-blue in lactoglycerol, steamed for 60 min, rinsed with tap water and stored in refrigerated cold water until microscopic examination. Cleared and stained root segments from each capsule were examined and tallied for the presence of arbuscular spores, vesicules and arbuscules of mycorrhizal fungi using a dissecting microscope and sub-sample with the compound microscope. Counts were tallied on a graduated petri dish.

Statistical Analyses

A statistical randomized block design and the analysis was performed utilizing ANOVA and Tukey's multiple range testing. Comparisons of

nursery seedling caliper growth, height, density, foliar phosphorous content and mycorrhizal colonization data were performed. Similarly, comparison of seedling caliper growth, height, survival, and mycorrhizal colonization data were compared by treatment for each of the two clearcut test sites.

Residuals from the data on caliper, height, and mycorrhizal colonization were plotted to determine if a log-normal transformation was called for to compensate for log-normally distributed values. This indeed was the case, so the data were accordingly transformed to produce a relatively normal distribution (Steel and Torrie 1960).

RESULTS

Nursery

After 5 months in the nursery, seedlings were evaluated for height, caliper growth, seedbed density, mycorrhizal colonization and foliar phosphorus levels (Figures 1-5).

MYCO/no P seedlings had significantly greater mycorrhizal colonization compared to all other treatments ($p<0.05$).

MYCO/P seedlings had significantly greater mycorrhizal colonization and height growth compared to No MYCO/P and No MYCO/No P treatments.

MYCO/No P and MYCO/P seedlings had significantly greater foliar phosphorous levels compared to No MYCO/P and No MYCO/No P treatments.

Outplanting Sites

After 14 months outplanted on the two clearcut sites, seedlings were evaluated for caliper growth, height growth, survival, and mycorrhizal colonization (Figures 6-9).

MYCO/no P and MYCO/P seedlings had significantly greater mycorrhizal colonization, caliper and height growth compared No MYCO/P and No MYCO/no P treatments at both clearcut sites ($p<0.05$).

MYCO/no P seedlings had significantly greater survival percentage compared to No MYCO/P and No MYCO/No P treatments at both sites.

MYCO/P seedlings did not significantly survive better than No MYCO/P seedlings at Clearcut Site 1 and No MYCO/no P seedlings at Clearcut Site 2.

MYCO/No P had significantly greater height growth, survival and mycorrhizal colonization than MYCO/P seedlings at Clearcut Site 1.

DISCUSSION

In this study, both Calocedrus growth and survival was influenced in both nursery and outplanting environment following AMF inoculation in biocided treated beds. Response was modified only slightly by the addition of phosphorous fertilizer at the nursery. Phosphorous addition in the MYCO/P treatment *did* significantly reduce the level of mycorrhizal colonization compared to the No MYCO/P treatment. However, even mycorrhizal colonization at 18% in the MYCO/P treatment was sufficient for the seedlings to significantly improve their growth performance and foliar phosphorous contents compared to the non-inoculated controls.

Young Calocedrus seedlings inoculated and colonized with AMF clearly produce more uniform seedlings with improved height and bed density compared to No Myco/P and No Myco/No P seedlings.

No Myco/P and No Myco/No P seedlings grew at lower densities and should have traditionally had, as a result, greater caliper and height growth. In this study the opposite was true, non-inoculated seedlings grew at low densities and had significantly less caliper growth and height.

Following fumigation, the addition of 200 pounds P fertilizer should have provided enough phosphate to the soil to saturate P-binding sites so that this essential nutrient would have been readily available to the roots. The bronzing effect and low foliar P level in the No MYCO/P treatment indicates that the higher level of phosphate was

apparently inadequate for sufficient P uptake when Calocedrus is non-mycorrhizal. The pre existing soil phosphorous levels were adequate for MYCO/No P to have sufficient foliar P levels for adequate growth even without the addition of P fertilizer.

At the seedling stage of plant growth, phosphorous uptake is presumably limited by the relatively small volume of soil occupied by root systems. AMF hyphae occupy a greater soil volume and produce specific enzymes for P extraction. In this study the presence of AMF significantly improved seedling P nutrition at the nursery.

While phosphorous is generally very mobile in plant tissue, the only phosphorous reserve in young seedlings comes from the seed itself. As seed reserves become exhausted the mycorrhizal association for P uptake is critical. Young seedlings, therefore may be more responsive to mycorrhizal colonization than older plants. Although both young and old plants require and benefit from the mycorrhizal association, the survival and growth response may be more dramatic for younger plants because of their poorly developed root systems.

High levels of soil phosphorous have been shown to reduce or eliminate mycorrhizal colonization of conifer species (Harley and Smith 1983.; Kough *et al.* 1985) found in a greenhouse study that AMF inoculated Thuja, Calocedrus, Sequoia and Sequoiadendron seedlings produced 100-2000% more biomass than non-inoculated seedlings at low P (11ppm) and from equality to 500% increase at higher P (43 ppm). In their study, AMF inoculation enhanced

seedling uniformity and size in all the tree species. Our results with Calocedrus support their findings in the more operational environments of a production nursery and reforestation sites.

Mycorrhizal colonization increased seedling uniformity as well as size. This uniformity effect of AMF inoculation has been reported on other host plants (Kough *et al.* 1985; Cooper 1981; Biermann and Linderman 1983). The economic benefit after fumigation is clear. Increased size and uniformity in the mycorrhizal treated beds means more seedlings acceptable for outplanting and less culled seedlings.

At both outplanting sites 1 and 2, results paralleled those at the nursery. MYCO/no P and MYCO/P seedlings clearly grew better compared to No MYCO/P and No MYCO/No P seedlings. At the Clearcut Site 1, however, the higher mycorrhizal colonization of MYCO/no P Calocedrus seedlings at outplanting apparently improved their survival and height growth when compared to MYCO/P seedlings. Numerous other studies have shown the effectiveness of AMF in promoting plant nutrition and establishment on tree hosts (Graham *et al.* 1982; Furlan *et al.* 1983; Amaranthus and Trappe 1993; Pattinson 2001a).

Still other studies have examined the use of AMF inoculum to encourage the re-establishment of postfire native vegetation. (Bellgard *et al.* 1994; Rashid *et al*. 1997 Pattinson *et al.* 2001a, 2001b). This study further supports the use of AMF inoculum on

disturbed sites to encourage plant establishment and early conifer growth.

Timber harvest and site preparation are the two most common and widespread deliberate forest activities in the Pacific northwestern United States. They significantly alter both the above and below ground environments. The outplanting test sites chosen were severely disturbed by clearcutting and the intense fire that accompanied the fall prescribed burn, which likely reduced indigenous AMF populations. Other studies have shown reductions in AMF activity following vegetation removal and intense fire.

Fourteen months after outplanting, the No MYCO/P and No MYCO/No P groups still had significantly lower mycorrhizal colonization, and less growth, than MYCO/P and MYCO/No P treatments. The two clearcut sites were prescribe burned and the intensity of the fire likely reduced the mycorrhizal colonization potential of the sites. Recent studies have examined the impact of wildfire and post-fire reestablishment (Amaranthus *et al.* 2004; Amaranthus and Trappe 1993; Vilarino and Arines 1991; Bellgard *et al.* 1994). This study's data indicate the two clearcut and prescribed burned outplanting sites had lost their ability to rapidly form mycorrhizae for outplanted seedlings. Where the mycorrhizal forming potential of a site has been reduced, mycorrhizal inoculation following fumigation may allow seedlings to more rapidly acquire site resources in the outplanted environment.

Many foresters have observed a significant lag in the growth of cedar seedlings following outplanting. In this study, mycorrhizal inoculated Calocedrus seedlings grew more rapidly in the field than non-inoculated nursery seedlings and thus may be a vital tool to encourage rapid growth of AMF host seedlings.

Summary

Mycorrhizal inoculation with Glomus intraradices following fumigation of nursery soils greatly enhanced Calocedrus decurrens performance at both the nursery and outplanting sites.

The response was modified only slightly by the addition of phosphorous fertilizer at the nursery. Phosphorous addition in the MYCO/P treatment significantly *reduced* the level of mycorrhizal colonization compared to the No MYCO/ No P treatment. However, even with the additional phosphorus treatment at the nursery, the seedlings average 18% mycorrhizal colonization root system performance and foliar phosphorous contents when compared to the non-inoculated controls.

Young Calocedrus seedlings inoculated and colonized with AMF clearly produce more uniform seedlings with improved height and caliper compared to No Myco/P and No Myco/No P seedlings. After 14 months planted in the clearcut sites, Calocedrus seedlings not inoculated at the nursery still had significantly less mycorrhzal colonization compared to nursery inoculated seedlings.

Increased seedling uniformity and size are tangible economic returns for the production nursery. Increased field survival and growth are important goals for foresters on difficult sites. Nursery practices such as using fumigants may produce non-mycorrhizal seedlings that perform poorly upon outplanting, especially on sites where the period for seedlings establishment is limited and native mycorrhizal colonization potential is low.

Table 1. Height (cm) of Calocedrus decurrens grown at J. Herbert Stone Nursery

Nursery Treatment	Ave seedling height at nursery (cm)
MYCO/no P	18.0 a
MYCO/P	14.4 b
No MYCO/P	11.2 c
No MYCO/No P	11.0 c

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 2. Caliper (mm) of Calocedrus decurrens grown at J. Herbert Stone Nursery

Nursery Treatment	Average seedling caliper (mm) at nursery
MYCO/no P	3.15 a
MYCO/P	2.55 b
No MYCO/P	2.58 b
No MYCO/No P	2.30 b

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 3. Seed bed density (every 30 cm) Calocedrus decurrens grown at J. Herbert Stone Nursery

Nursery Treatment	Average bed density at nursery (every 30 cm)
MYCO/no P	46.0 a
MYCO/P	34.2 b
No MYCO/P	26.8 c
No MYCO/No P	14.4 d

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 4. Percent mycorrhizal colonization of Calocedrus decurrens grown at J. Herbert Stone Nursery

Nursery Treatment	Percent mycorrhizal colonization
MYCO/no P	38.0 a
MYCO/P	18.2 b
No MYCO/P	0
No MYCO/No P	0

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 5. Percent foliar phosphorous level of Calocedrus decurrens grown at J. Herbert Stone Nursery

Nursery Treatment	Percent P^2O^5
MYCO/no P	0.14 a
MYCO/P	0.14 a
No MYCO/P	0.07 b
No MYCO/No P	0.05 b

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 6. Caliper growth (mm) 14 months following outplanting

Nursery Treatment	Clear cut A	Clear cut B
MYCO/no P	1.45 a	1.38 a
MYCO/P	1.28 a	1.69 a
No MYCO/P	0.70 b	0.45 b
No MYCO/No P	0.65 b	0.54 b

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 7. Height growth (cm) 14 months following outplanting

Nursery Treatment	Clear cut A	Clear cut B
MYCO/no P	6.3 a	5.2 a
MYCO/P	5.1 b	4.6 a
No MYCO/P	2.5 c	2.7 b
No MYCO/No P	2.2 c	2.3 b

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 7. Survival percentage 14 months following outplanting

Nursery Treatment	Clear cut A	Clear cut B
MYCO/no P	79.5 a	69.8 a
MYCO/P	60.2 b	67.2 ab
No MYCO/P	51.2 bc	50.0 c
No MYCO/No P	44.4 c	53.2 bc

Alpha symbols denote statistically significant different results ($p<0.05$)

Table 8. Mycorrhizal colonization percentage 14 months following outplanting

Nursery Treatment	Clear cut A	Clear cut B
MYCO/no P	41.8 a	49.0 a
MYCO/P	30.2 b	56.4 a
No MYCO/P	19.0 c	21.4 b
No MYCO/No P	14.7 c	24.2 b

Alpha symbols denote statistically significant different results ($p<0.05$)

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