Inoculation of Fumigated Nursery Beds and Containers With Arbuscular Mycorrhizal Products for Eastern Redcedar Production

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Abstract

Commercially available arbuscular mycorrhizal (AM) products were applied at an operational rate to eastern redcedar (*Juniperus virginiana* L.) nursery beds and containers to evaluate seedling growth and colonization responses. A field study at the Augusta Forestry Center in Crimora, VA, and a companion container study were initiated in the fall of 2012. MycoApply® Endo products containing the same four species of AM fungi were applied as a liquid, granular, or seed treatment. The field application of AM products did not result in early root colonization by AM fungi. By November 2013, seedlings were colonized by naturally occurring AM fungi and seedlings did not differ in size among treatments. A winter rye cover crop treatment tested in conjunction with the AM treatments in the container study did not significantly affect AM colonization. AM colonization of seedling roots was very low in container seedlings from all treatments and no growth response could be attributed to AM fungi. This paper was presented at a joint meeting of the Northeast Forest and Conservation Nursery Association and Southern Forest Nursery Association (Williamsburg, VA, July 21–24, 2014).

Introduction

Many forest nurseries in the South grow eastern redcedar (*Juniperus virginiana* L.) as a minor use specialty crop. Seedlings of eastern redcedar are commonly used in the South for establishing Christmas tree farms, wildlife habitat areas, windbreaks, and other soil stabilization projects. Growing this conifer species unfortunately has presented challenges for nursery managers. One of the most documented causes of redcedar seedling losses has been Phomopsis blight, caused by the fungus *Phomopsis juniperovora* Hahn (Otta et al. 1980). Stunting also results in poor crops in which no biological pests are found (figure 1). One theory regarding the cause of periodic stunting in eastern redcedar is that fumigation before sowing removes the arbuscular mycorrhizal (AM) fungi from the seedling root zone.

Figure 1. Eastern redcedar stunting due to unknown causes. (Photo by Michelle Cram, 2009)

Arbuscular mycorrhizae are the result of a symbiotic association between an endomycorrhizal fungus and a plant root. AM fungi take carbon from the plant host and increase nutrient uptake and drought tolerance of the host (Allen et al. 2003). The presence of AM roots on eastern redcedar is believed to enhance the ability of this species to thrive under low-fertility environments (Williams et al. 2013). The effects of fumigation and AM colonization on eastern redcedar growth in nurseries have not been studied; however, applications of AM-type mycorrhizae mixtures can increase seedling growth of cade juniper (*Juniperus oxycedrus* L.) (Alguacil et al. 2006). There is also evidence that many other tree species dependent on endomycorrhizae have reduced growth when AM fungi are absent or colonization is delayed (Berch et al. 1991; Bryan and Kormanik 1977; Douds and Chaney 1982; Kormanik et al. 1977, 1982). Commercial AM inoculants are available for use in forest nurseries and are being used on a routine basis for some species (Amaranthus and Steinfeld 2005, Carpio et al. 2003, Meikle and Amaranthus 2008). Tests of various commercially available AM products have shown that seedling growth responses can be positive to AM inoculations; however, they also show a high degree of specificity between individual host species and the AM product applied (Carpio et al. 2003, Corkidi et al. 2005). From these few studies, it is evident that

managers should test AM products on a given species before operational use to determine if the product provides enough benefit to warrant the cost.

The purpose of this study was to evaluate commonly used mycorrhizal products on eastern redcedar in a forest nursery field site at operational application rates. A container study was also conducted in a growth chamber under similar rates and conditions as the field study. The nursery that participated in this study uses a winter rye (*Secale cereale* L.) cover crop to protect seedbeds from frost heaving and other severe weather conditions. Because of this practice, a winter rye cover crop treatment was also added to the container study to evaluate potential effects on AM colonization of seedling roots and subsequent growth response of seedlings.

Methods

Field Study

Several nursery beds were used at the Augusta Forest Center Nursery in Crimora, VA, to evaluate three formulations of MycoApply® Endo products (Mycorrhizal Applications, Inc., Grants Pass, OR) applied to soil or to seed before sowing the beds with eastern redcedar. A control treatment was also established for comparison. Seeds were obtained from the F.W. Schumacher Company (Sandwich, MA) and were from an Eastern U.S. coastal source. The field soil was a loam (46:32:22 sand:silt:clay) with 2.7 percent organic matter. The study area was fumigated in October 2012 at 400 lb/ac (448 kg/ha) with 80:20 methyl bromide:chloropicrin. Rye seed used as a cover crop was from Discount Seeds (Watertown, SD, Lot 12232).

On October 25, 2012, three commercial formulations of MycoApply® (table 1) were applied to the field or to the seeds just before sowing. Treatment plots were 4.00 ft wide by 30.00 ft long (1.22 m by 9.15 m), and each treatment was replicated four times in a randomized complete block design. The AM species listed on the labels of all three MycoApply® products used in this study have been recently undergoing taxonomic reclassifications. According to Redecker et al. (2013), the Schüßler and Walker (2010) taxonomic treatment of the AM species is generally accepted for this group of fungi and, therefore, will be the primary authority to name the AM species used in this study. Each MycoApply® formulation included equal parts of *Glomus aggregatum* Schenck and Smith, *Funneliformis mosseae* (= *G. mosseae* Nicol. and Gerd.), *Rhizophagus intraradices* (= *G. intraradices* Schenck and Smith), and *Claroideoglomus etunicatum* (= *G. etunicatum* Becker and Gerd.). The application rate for all three products was based on the recommended field rate for the Liquid Endo at 3.0 gal (11.3 L), or 10.8 million AM propagules, per 100,000 seedlings. Eastern redcedar was sown at a rate to obtain a seedling density of approximately 10.0 seedlings/ft^2 (107.5/m^2), and, therefore, the liquid and granular formulations were applied at 1,080 AM propagules/ft^2 (11,613 propagules/m^2) surface area and rototilled into the soil. The seed treatment was applied at a rate equivalent to 1,080 AM propagules/ft^2 (11,613 propagules/m^2) by mixing 68.0 ml (2.3 oz) of the AM seed treatment with 1.20 lb (0.54 kg) of eastern redcedar seeds, which was sown over 480.0 ft^2 (44.6 m^2). The winter rye was sown at 1.23 lb/480.0 ft^2 (0.56 kg/44.6 m^2) in all the treatments.

Table 1. Three commercial arbuscular mycorrhizal (AM) formulations of MycoApply® evaluated for eastern redcedar seedling production.

MycoApply® AM product	AM propagules	Cost in 2012
Liquid Endo	3,600,000 propagules/gal	$619/gal
Endo Granular	60,000 propagules/lb	$6.49/lb
Liquid Endo (Seed & Furrow)	3,600,000 propagules/16 oz	$619/16 oz

Nursery personnel applied glyphosate on March 23, 2013, to kill the winter rye. Beginning May 30, fertilizer was applied at 100 lb/ac (112 kg/ha) every 2 weeks as a liquid until mid-August. The fertilizer applications were alternated between formulations of 30 percent nitrogen, and an 8 percent sulfur + 9 percent nitrogen fertilizer mix. Pesticide applications included a Pyrethrin application on April 3, 2013, and prothioconazole applications beginning April 3, 2013, and rotated with thiophanate-methyl every 2 to 3 weeks, as weather permitted, throughout the summer for control of Phomopsis blight.

On June 13, 2013, 8 weeks after emergence, redcedar seedling density was determined. Three subplots per treatment plot were counted using a 1.0 by 4.0 ft (0.3 x 1.2 m) frame. Ten seedlings per plot were collected randomly in between counting frames to assess mycorrhizal colonization of roots. Samples were placed in a cooler and kept at 40 °F (4 °C) until processed. In the laboratory, the AM root colonization was assessed by clearing and staining roots with a modified procedure outlined by Kormanik et al. (1980a). Roots from the 10 seedlings in each treatment plot were cut into 0.59 in (1.50 cm) pieces. A 0.05 oz (1.50 g) subsample of roots from the ten seedlings was soaked in 10% (w/v) KOH for 60 minutes at 194 °F (90 °C). Roots were then soaked for 60 min in an alkaline hydrogen peroxide solution (3 ml of NH_4OH and 30 ml of 10% H_20_2 in 567 ml of water) for additional clearing. Cleared roots were rinsed for 3 min in 1% HCL solution then stained with Trypan Blue at 0.05% (w/v) for 20 minutes at 194 °F (90 °C). Stained root samples were then destained with lactoglycerol for 24 hr (Brundrett et al. 1984). The frequency

of AM colonization for each plot was estimated using the gridline intersect method (Giovannetti and Mosse 1980). The percentage of mycorrhizal root colonization was calculated as the number of intersects in which AM fungal structures were present divided by the total number of intersects examined. The mean AM colonization rate for each plot was based on the average of three sets of observations for each 0.05 oz (1.50 g) root sample.

On November 5, 2013, 20 seedlings were lifted from 4 to 5 areas of each plot and placed in a cooler, where they were maintained at 40 °F (4 °C) until processed. For each plot, 10 seedlings with less than 10 percent foliar damage caused by Phomopsis blight were measured for height, root-collar diameter, and shoot and root fresh and dry weights. A single 0.035 oz (1.000 g) composite root subsample was removed from the 10-seedling sample to determine the AM infection rate; the remaining roots were dried for 48 hours at 176 °F (80 °C). The average root dry weights were based on the combined dry weight of the 10 seedling roots for each plot plus the estimated dry weight of the root subsample. The dry weight of each root subsample was estimated using the fresh weight to dry weight ratio of the root sample for each plot.

Root samples for AM assessments were cleared and stained using the same process as described previously. Because of difficulties in reading roots at the 12x magnification level used for the gridline intersect method, final readings were made using a slide intersect method (McGonigle et al. 1990). Ten 0.59-in (1.50-cm) root segments were placed lengthwise on a slide in lactophenol with a cover slip and sealed with clear nail polish. Three slides were prepared per root sample. A compound microscope at 200x magnification with a hairline graticule was used to make 4 passes across each slide until 150 intersections were examined for AM structures for an estimated percentage of root lengths colonized by AM fungi.

Container Study

A companion container study was initiated in a growth chamber on November 2, 2012. The container study was established as a 2 by 4 factorial design, replicated 4 times, with 2 cover crop treatments (with and without winter rye) and 4 AM treatments. The same 4 AM treatments used in the field study were applied at the same rates, with the liquid and granular products applied at 1,080 AM propagules/ft^2 (11,613 propagules/m^2) surface area and the seed treatment applied at 108 propagules/seedling (5 seedlings/container).

Soil for the container study was collected from the fumigated field used for the study at the Augusta Forest Center Nursery. Soil was put through a soil sieve No. 10 with 0.078 in (1.981 mm) openings to break up clods. Soil for each container was sterilized individually by adding 100 ml H_2O to 88.2 oz (2,500.0 g) of soil (moisture content of 8.8 percent) and microwaving for 8 min (Ferriss 1984). The microwaved soil reached temperatures of approximately 200.0 °F (93.4 °C), and the soil was allowed to cool for 24 hr before the addition of AM treatments. Each 6.0-in-deep (15.2-cm-deep) container (28.2 in^2 [182.3 cm^2] surface area) was filled with the 88.2 oz (2,500.0 g) of soil. The granular and liquid AM treatments were applied at approximately 212 AM propagules/container by mixing the treatments into the sterilized bag of soil before placing the treated soil in the container. The seed treatment was applied at 108 AM propagules/seedling by mixing 0.019 oz (0.560 ml) with 168 seeds and sowing 21 seeds/container, which was later thinned to 5 seedlings/container. Winter rye was sown at a rate of 0.007 oz (0.220 g)/container and sterilized vermiculite was then placed on top of the seeds at a depth of 0.39 in (1.00 cm). The maximum and minimum daily temperatures maintained in the growth chamber during the study were designed to mirror the seasonal pattern at the Augusta Forest Center Nursery (figure 2). Glyphosate was applied by a brush to the winter rye just before seedlings began to emerge on March 24, 2013. Nitrogen (NH_4NO_3) was applied at 0.018 oz/ft^2 (5.493 g/m^2) on July 8, July 29, August 19, and September 13. The last fertilization was applied on October 29, 2013, at 0.016 oz/ft^2 (4.882 g/m^2).

On December 9, 2013, seedlings were removed from containers by soaking them in water and gently washing soil from the root systems. Seedling height, root-collar diameter, and shoot and root fresh and dry weights were determined as previously described for seedlings in the field study. Methods for subsampling roots and determining the AM infection rate were also the same as described for the field study.

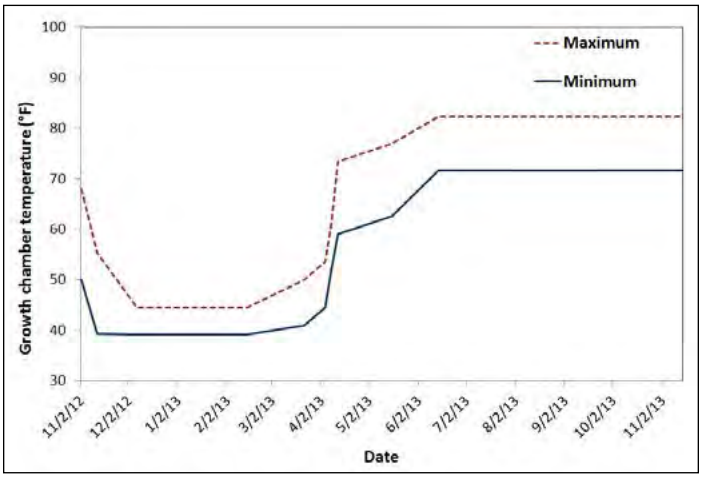

Figure 2. Minimum and maximum temperatures in growth chamber for container study.

Statistical Analyses

All data were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test (Systat 13, Systat Software, Inc., Chicago, IL). All data, except the AM root colonization data in the container study, were statistically analyzed by ANOVA, using the PROC GLM procedure of SAS software (SAS Institute, Inc., Cary, NC). Mean separation was performed by Tukey's HSD test. Data from the field study were analyzed as a randomized complete block design, while the container data was analyzed as a 2 by 4 factorial completely randomized design. The percentage of AM root colonization in the container study was transformed by arcsine (sqrt(X)) before analysis, but this transformation failed to provide equal variances among the mycorrhizal treatments. The data for the percentage of AM root colonization for treatment and cover crop effects were subsequently analyzed by nonparametric statistics using the Kruskal-Wallis test, and mean separation was performed by the Dwass-Steel-Chritchlow-Fligner Test for all pairwise comparisons (Systat 13, Systat Software, Inc., Chicago, IL).

Results

Field Study

The germination rate of the eastern redcedar was approximately 46 percent, 4.6 times greater than expected; therefore, the inoculation rate per seedling was actually 23 propagules/seedling.

AM root length colonization 8 weeks after emergence was extremely low (0–0.83 percent), with no significant differences among treatments. By the November sampling, 23 to 54 percent root colonization by AM fungi occurred in all treatments (table 2), but there were no differences among treatments. Similarly, final seedling morphology did not differ significantly among treatments (table 2).

Container Study

Seedling height, root-collar diameter, and dry weight were significantly affected by AM and cover crop treatments in the container study, but no interactions occurred between the treatment factors for any of the seedling parameters (table 3). Although seedling size was significantly lower for seedlings sown with winter rye, AM colonization of roots did not appear to be affected by the cover crop (table 4). Mean AM root colonization among mycorrhizal treatments ranged from 2.2 to 11.8 percent with the rye cover crop and from 2.8 to 9.7 percent with no rye cover crop. The granular formulation and the liquid formulations significantly increased AM root colonization compared to the control treatment (table 5). None of the AM treatments significantly affected seedling growth compared with the control. Seedlings in the AM seed treatment had increased shoot growth compared with those in the granular or liquid treatments, although AM root colonization was not greater in the seed treatment compared with the control (table 5).

Table 2. Morphology and arbuscular mycorrhizal (AM) colonization of seedlings in the field study, November 5, 2013.[1]

MycoApply® treatment	RCD (mm)	Height (mm)	Shoot dry weight (g)	Root dry weight (g)	Percent AM colonization[2]
Granular	3.00 a	190.38 a	1.96 a	0.48 a	54.0 a
Liquid	2.79 a	163.25 a	1.42 a	0.43 a	38.0 a
Seed treat	2.69 a	154.63 a	1.35 a	0.39 a	23.2 a
Control	2.73 a	181.75 a	1.45 a	0.44 a	30.3 a

RCD = root-collar diameter.

[1] Means within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to Tukey's studentized range test.

[2] Percent root length colonized by AM fungi.

Table 3. Results of statistical analyses (p-values) of arbuscular mycorrhizal (AM) treatments and cover crop and their interactions on eastern redcedar morphology and root colonization in the container study.[1]

Source of variation	RCD	Height	Shoot dry weight	Root dry weight	AM colonization
AM treatment	0.008	0.007	0.020	0.205	0.0101
Cover crop (CC)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0642
AM x CC	0.792	0.883	0.868	0.512	—

RCD = root-collar diameter.

[1] All variables analyzed by analysis of variance, except the AM colonization, which was analyzed by the nonparametric Kruskal-Wallis test.

Table 4. Morphology and arbuscular mycorrhizal (AM) root colonization of seedlings in the container study as affected by cover crop treatment after 7 months.[1]

Cover crop	RCD (mm)	Height (mm)	Shoot dry weight (g)	Root dry weight (g)	AM colonization[2, 3] (%)
Control	2.51 a	136.89 a	1.21 a	0.85 a	4.41 a
Winter rye	1.48 b	86.58 b	0.37 b	0.33 b	6.38 a

RCD = root-collar diameter.

[1] Means followed by the same letter are not significantly different ($p \leq 0.05$) according to Tukey's studentized range test.

[2] Means within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to the nonparametric Kruskal-Wallis test.

[3] Percent root length colonized by AM fungi.

Table 5. Seedling morphology and arbuscular mycorrhizal (AM) colonization of seedlings with different MycoApply® formulations in the container study.

MycoApply® treatment	RCD[1] (mm)	Height[1] (mm)	Shoot dry weight[1] (g)	Root dry weight[1] (g)	AM colonization[2, 3] (%)
Granular	1.80 b	99.83 b	0.67 b	0.52 a	10.75 a
Liquid	1.84 b	108.26 ab	0.69 b	0.55 a	8.26 a
Seed treat	2.36 a	130.68 a	1.09 a	0.72 a	2.50 ab
Control	1.97 ab	108.16 ab	0.71 ab	0.59 a	0.17 b

RCD = root collar diameter.

[1] Means within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to Tukey's studentized range test.

[2] Means within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to the Dwass-Steel-Chritchlow-Fligner test.

[3] Percent root length colonized by AM fungi.

Discussion

The AM fungi in the MycoApply® Endo products did not provide a growth benefit to eastern redcedar at the rate applied in the field or in the container studies. In the field study, the colonization of less than 1 percent of roots by AM fungi of the 8-week-old seedlings not only indicated that the AM inoculation was ineffective but also that naturally occurring AM fungi were reduced by fumigation in the soil's seed germination zone. The use of a winter rye as a living mulch in the seedbeds was expected to increase the inoculum potential of the endomycorrhizae (Kabir and Koide 2002, Kormanik et al. 1980b). In both the field and container studies, any increase in AM inoculum that may have occurred from the presence of winter rye was not enough to significantly affect early root colonization. Natural AM inoculum populations in the field increased over the summer and fall, and colonized all seedlings by the time of lifting, including those in the control treatments, and did not differ among treatments. This recolonization by AM fungi after fumigation is common, as viable AM fungi can remain in the soil profile outside the effective fumigation zone (An et al. 1990, Barnhill 1981, Snyder and Davey 1986). The problem with late AM root colonization is that it can be unevenly distributed within root systems and among seedlings, and many seedlings may remain stunted for a considerable time well into the growing season (Snyder and Davey 1986, South 1977).

The only mycorrhizal treatment that appeared to affect seedling growth was the seed treatment in the container study. The lack of a corresponding increase in AM root colonization indicates that AM fungi were not the cause for the increased seedling growth. This unexpected result suggests that the seed treatment may have contained something besides AM fungi that could stimulate shoot growth. The only other growth response was the smaller seedlings in the winter rye treatment, which was likely due to an inhibitory effect from the rye residue decomposing during germination and early seedling growth (Bonanomi et al. 2011).

The container studies demonstrated that at least one of the AM species in the MycoApply® Endo products can colonize eastern redcedar (figure 3), but it is unclear if this species can provide a benefit to the seedling. The granular formulation and the liquid formulation resulted in significant root colonization by AM, but the level of colonization was below 11 percent.

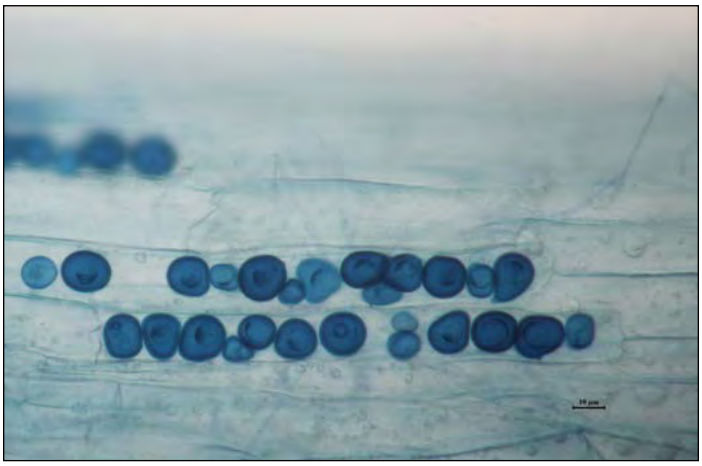

Figure 3. Arbuscular mycorrhizae roots from containerized eastern redcedar inoculated with MycoApply® granular formulation study. (Photo by Michelle Cram, 2014)

The lack of a growth response associated with AM treatments in these studies was most likely due to the low root colonization rate by the AM fungi. Other studies using similar commercial MycoApply® products applied to other host species have had better AM root colonization and significant seedling growth responses, but some key differences existed in application methods or AM fungi species used, which may affect the results. The application of an AM fungus to incense-cedar (*Calocedrus decurrens* Torr.) in an Oregon nursery used the same rate of application as in our study, but only one AM species (*Rhizophagus. intraradices*) was present (Amaranthus and Steinfeld 2005). By using a single, effective AM species, the application rate was, in effect, 4 times our application rate of the same species. In another study, the same MycoApply® Endo product containing a single AM species was used on sweetgum (*Liquidambar styraciflua* L.), but the application was applied directly to the roots of 8-day-old germinating seedlings, resulting in a high root colonization rate after 8 and 14 weeks (Corkidi et al. 2005).

The application rate of AM propagules in the field study was lower on a per-seedling basis than intended because of a higher germination rate, but not on an area basis. The lack of a seedling growth response to the MycoApply® Endo liquid and granular formulations in the container study suggests that the field results would probably not have been different even if the germination rate had resulted in the expected 10.0 seedlings/ft^2 (107.6/m^2). Given that the AM treatments were quite expensive, the use of a higher application rate would most likely be cost prohibitive at most nurseries. One way to increase AM product effectiveness could be to manipulate the application method and maximize seedling root contact with the inoculant. Application of MycoApply® on sweetgum by Corkidi et al. (2005) directly to the roots at 8 days produced AM colonization of 41 to 79 percent at 8 and 14 weeks, respectively. The granular and liquid formulations could be more effective by concentrating the product directly within the seed row or directly below the seed in nursery beds. Additional studies are needed to determine the AM species best suited for eastern redcedar seedlings, and to evaluate the effects of high root colonization rates from endomycorrhizal fungi.

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