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Mosaic Stunting in Jack Pine Seedlings in a Northern Michigan Bareroot Nursery

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Abstract: Mosaic, or patchy, stunting of bareroot conifer seedlings is thought to be caused by deficiencies of mycorrhizal fungi following fumigation, resulting in reduced nutrient uptake, particularly phosphorus. Mosaic stunting of jack pine (*Pinus banksiana*) seedlings was observed in 2005 at the USDA Forest Service JW Toumey Nursery in Watersmeet, MI. We initiated a study to determine if either the species of mycorrhizal fungi or the quantity of mycorrhizae were different on stunted and healthy seedlings. In 2006 and 2007, we tested the soil, sampled root tips, and analyzed seedling growth and foliar nutrient concentrations. In 2006, we used DNA sequencing to identify mycorrhizal fungi. Our results showed four main fungal taxa (*Sistotrema brinkmannii*, *Thelephora terrestris*, *Suillus luteus*, and *Laccaria* spp.) were associated with mycorrhizal root tips on stunted and healthy seedlings, and that the number of mycorrhizal root tips was high on both types of seedlings, although we observed variation among stocktypes and years. Despite soils having similar nutrient concentrations, we observed differences in foliar concentrations between stunted and healthy seedlings. Only 1+0 seedlings in 2007, however, showed significant differences. This suggests that reduced root nutrient uptake was a factor in stunting of 1+0 seedlings in 2007, but does not appear to be related to ectomycorrhizal fungi.

Keywords: ectomycorrhizal fungi, foliar nutrients, bareroot, sawdust, organic matter, *Sistotrema brinkmannii*, *Thelephora terrestris*, *Suillus luteus*, *Laccaria* spp.

Mosaic-pattern stunting is identified as random patches of seedlings with reduced top and root-collar diameter growth and chlorosis. Mosaic-pattern stunting has been observed in a variety of tree species in bareroot and container nurseries (Landis 1998). In container nurseries, stunting is most often associated with compacted growing media; in bareroot nurseries, the cause is often more vexing. Stunted bareroot seedlings that lack biotic disease (Tanaka and others 1986; Linderman and others 2007), combined with variation in soils and nursery practices, often make finding a conclusive cause difficult (Landis 1998). In most cases, the problem is thought to be linked to a lack of ectomycorrhizal fungi in the nursery soil caused by soil fumigation (Campagna and White 1969; Trappe and Strand 1969; Henderson and Stone 1970).

Fumigation eliminates ectomycorrhizal fungi (Henderson and Stone 1970; Ridge and Theodorou 1972). Because ectomycorrhizae are important for nutrient uptake in pines (McComb 1938; Trappe and Strand 1969), this lack of a symbiotic association results in poor seedling growth (Henderson and Stone 1970; Croghan and others 1987). Nursery soil is recolonized by ectomycorrhizal fungi in a random pattern. These pockets of mycorrhizae provide better access to nutrients, and seedlings grow better than their non-inoculated cohorts, resulting in random patches of excellent seedling growth interspersed with stunted growth (Landis 1998). Most studies with mosaic stunting have, therefore, focused on ectomycorrhizae levels (Croghan and others 1987; Linderman and others 2007).

In 2005, mosaic stunting was identified in jack pine (*Pinus banksiana*) seedlings at the USDA Forest Service JW Toumey Nursery, located in the Upper Peninsula of Michigan. We investigated potential causes of stunting. We compared mycorrhizal fungi (species and the number of mycorrhizal root tips) and soil nutrients for stunted and healthy seedlings. Our goal was to assist nursery personnel in identifying the cause of stunting in jack pine seedlings, and thereby ameliorate future incidence.

Methods

The USDA Forest Service JW Toumey Nursery is located on the Ottawa National Forest in Watersmeet, MI (46°27' N; 89°17' W). The soil is a fine sandy loam soil of the Pence-Vilas complex (NRCS 2007). The primary stocktypes produced are jack pine, red pine (*Pinus resinosa*), and eastern white pine (*P. strobus*). Mean annual precipitation is 77 cm (30 in) and mean annual air temperature is 4 °C (39 °F) (MCRP 2009).

Soil Sampling and Seedling Measurements

In 2006 and 2007, we selected 10 plots within areas of mosaic stunting of jack pine seedlings. These plots were selected throughout the length of a field, and included 1+0, 2+0, and, in 2006, some 3+0 holdover stock. Each plot had at least 10 stunted seedlings adjacent to healthy seedlings. From each plot, we extracted eight soil cores (four under stunted and four under normal seedlings) to a 15-cm (6-in) soil depth with a 2-cm (0.8-in) diameter soil probe, and sampled 5 stunted and 5 healthy seedlings. Samples were refrigerated at 2 °C (36 °F) until processed.

Soil samples were dried at 105 °C (221 °F), sent to the Rocky Mountain Research Station in Moscow, ID, and analyzed for pH, total carbon (C) and nitrogen (N), available phosphorus (P), and exchangeable potassium (K), calcium (Ca), and magnesium (Mg). Exchangeable K, Ca, and Mg were extracted with pH neutral ammonium acetate and processed on a Perkin Elmer® Atomic Absorption Spectrometer (Model 5100PC). Available P was estimated using the Bray 1 method and analyzed on an OI Analytical Flow Solution® 3000 (College Station, TX); C and N were analyzed on a LECO CN2000 (LECO Corporation, St Joseph, MI). Soil pH was measured in a 1:2 (v:v) soil:deionized water slurry.

Seedlings were carefully rinsed. Root sections for mycorrhizae analysis were removed from seedlings and stored in water at 2 °C (36 °F) less than 24 hours before analysis. We measured seedling height and root-collar diameter. Shoots (needles and stems) and roots of 1+0 seedlings (sampled in September 2007) and needles of 2+0 seedlings (July 2006, July 2007, August 2007) were ground with a Wiley Mill to pass a 40-mesh screen and analyzed for nutrient concentrations at the Penn State Agricultural Analytical Resources Laboratory (University Park, PA). Total N was determined on a Carlo Erba NA1500 Elemental Analyzer (Horneck and Miller 1998). Analysis was done for P, K, Ca, Mg, manganese (Mn), iron (Fe), copper (Cu), boron (B), sodium (Na), and zinc (Zn) after dry ashing at 500 °C (932 °F) (Miller 1998).

Mycorrhizae Quantification, Isolation, and Identification

To quantify mycorrhizae, we excised a 3-cm (1.2-in) section of root from the third and sixth lateral roots of each seedling. Using a dissecting microscope (10x to 40x power), we counted mycorrhizal root tips on each root section and categorized by morphology. Using sterile technique, we then isolated the fungi from mycorrhizal root tips within 24 hours of sampling to improve success rate (Molina and Trappe 1982). A 2% malt (2M) agar medium with additions of antibacterials (100 ppm streptomycin [S] and 100 ppm tetracycline [T]) and 10 ppm of the fungicide benomyl (B) (Benlate®) (2MSTB) was used for isolations and culturing. For root tip isolations, we clipped three 3-cm (1.2-in) sections from the lateral roots of one stunted and one healthy seedling per plot. Excised roots were surface sterilized for 30 seconds with agitation using a 1:10 (v:v) Clorox® solution (5.25% sodium hypochlorite) water solution, and rinsed three times with sterile water (slightly modified from methods of Zak and Bryan 1963). Two mycorrhizal root tips were removed from each 3-cm (1.2-in) root section and plated on Petri dishes. For each sample date, we used approximately 120 total root tip pairs (10 plots x 4 seedlings [two stunted; two healthy] x 3 root sections).

Possible ectomycorrhizal fungi are characterized by slow hyphal growth, presence of clamp connections on hyphae, and distinctive colony characteristics (Zak and Bryan 1963); we transferred cultures with these characteristics to fresh MMN (modified Melin-Norkrans) agar (Marx 1969) for further study. After at least 3 weeks of growth at 26 °C (79 °F), cultures having at least 15 hyphae were organized by "type" based on macro- and micro-morphological characteristics (Hutchison 1991). When characterized, the fungal isolates were actively growing for 3 weeks at 26 °C (79 °F) and a minimum of 15 hyphae per type. In 2006, we identified these types to species using DNA sequencing as described in Potvin (2008).

Statistical Analysis

We used a two-tailed paired-sample t-test to look for differences between stunted and healthy seedlings for each year x stocktype x sample date combination for these variables: 1) seedling morphology (height [n = 50]; RCD [n = 50]); 2) mean foliar nutrient concentrations; 3) mean soil nutrient concentrations from soils beneath sampled seedlings (n = 20); 4) mean numbers of monopodial, bifurcate, dichotomous, coralloid, and pinnate root tips morphologies (2006 seedlings; n =120), and 5) total mycorrhizal root tip counts (2006 seedlings; n = 120). Chi-square

Table 1. Stunted and healthy seedling morphology measurements taken in 2006 and 2007.

		2006			2007	
		3+0	2+0	1+0	2+0	1+0
Height (cm)*	Healthy	19.90	23.15	5.25	17.25	7.40
	Stunted	7.50	7.20	1.55	5.20	1.10
	P value[2]	0.000	0.000	0.000	0.000	0.000
Root Collar Diameter (mm)	Healthy	4.15	3.99	NA[1]	3.35	1.30
	Stunted	2.31	1.79	NA[1]	2.00	0.61
	P value[2]	<0.0001	<0.0001	NA[1]	<0.0001	<0.0001

[1] RCD measurements were not measured on these seedlings as all calipers were <1.0 mm.
[2] Values < 0.05 are statistically different.
* 1 cm = 0.4 in

goodness of fit was used to analyze differences between stunted and healthy seedlings for specific fungal types isolated from root tips of stunted and healthy seedlings in 2006. Tests were considered significant if $P < 0.05$.

Results and Discussion

Seedlings and Soil

Stunting was most prevalent in 1+0 jack pine seedlings in 2007, with widespread growth deficiencies and chlorosis. Stunted seedlings were significantly shorter with significantly less stem diameter (Table 1). Root dry weights of healthy 2+0 seedlings were 189% greater than stunted seedlings in 2007, but were not significantly different in 1+0 seedlings. For shoots, healthy 1+0 and 2+0 had 280% and 267% greater biomass, respectively, than stunted seedlings in 2007.

For both years, we found no significant differences in extractable Ca, K, Mg, total C and N, available P, and pH in the soil below either stunted or healthy seedlings. Concentrations of N, P, K, Mn, and Zn in shoots and roots of stunted 1+0 seedlings sampled in 2007 were 159%, 133%, 27%, and 86%, respectively, lower than in healthy seedlings, while Ca and Mg were 169% and 36% greater in stunted seedlings. In contrast, N and P concentrations in stunted and healthy 2+0 seedlings were not significantly different in 2006 and 2007. Because most researchers suspected a mycorrhizae deficiency, the focus of their work has been on P nutrient contents; lower P values have been observed by others in stunted seedlings (Campagna and White 1969; Trappe and Strand 1969; Croghan and LaMadeleine 1982).

The N, P, and K levels observed in the stunted 1+0 seedlings were consistent with other studies, and just below, or at the low end of, the acceptable range of values for bareroot seedlings (Table 2). Additional soil nutrient analysis, involving available N, may provide further insight into seedling nutrient deficiencies. Applications of foliar $21N{:}0P_2O_5{:}0K_2O$ and $19N{:}19P_2O_5{:}19K_2O$ fertilizers to 2+0 stunted seedlings did result in increased growth when compared to stunted seedlings with no fertilizer. When the heights were compared to control healthy seedlings, however, the seedlings were still not up to grading specs (Koll 2008). Applying fertilizer as a top dressing when stunting first appears could ameliorate growth deficiencies, but this has not been tested at this time. Average soil pH ranged from 4.68 to 4.94. This is acceptable, but not ideal, for soil nutrient conditions. Slightly raising soil pH could also improve nutrient availability and possibly reduce seedling growth deficiencies in jack pine.

Mycorrhizae

Healthy 2+0 and 3+0 seedlings had significantly more mycorrhizal root tips than stunted seedlings, but no significant difference was detected in 1+0 seedlings (Table 3). While 2+0 and 3+0 stunted seedlings had statistically less mycorrhizal root tips, the total numbers were still very high when compared to healthy seedlings, and stunted seedlings did not appear to be deficient in mycorrhizae. In 2007, visual estimates of colonization by mycorrhizal fungi on fresh 1+0 and 2+0 seedling roots indicated no major differences between stunted and healthy seedlings.

We classified seedlings from 2006 into three distinct types of ectomycorrhizal fungi. We compared the DNA sequences with known sequences in the National Center for Biotechnology Information BLAST search and the UNITE database (Kõljalg and others 2005) (see Potvin 2008 for details). Our three distinct types were identified as *Sistotrema brinkmannii* (Bres.) J. Erikss., *Thelephora terrestris* Ehrh., and *Suillus luteus* (L.: Fries) Gray. The fungi we isolated in 2007 had similar growth characteristics in culture as these three types. In 2007, we also identified a fourth type as a *Laccaria* spp. using comparisons with known pure cultures of *Laccaria laccata* ((Scop.) Cooke.).

All but *S. brinkmannii* are mycorrhizal fungi common to conifer nursery systems (Trappe and Strand 1969; Croghan 1984; Richter and Bruhn 1993; Menkis and others 2005). *S. luteus* was the most frequently isolated mycorrhizal fungus in this study, and commonly colonize pine nursery seedlings in their first months of growth (Richter and Bruhn 1993; Dahlberg and Finlay 1999). *T. terrestris* was also detected on almost all seedlings, and is an aggressive colonizer of pine seedling roots in nurseries (Richter and Bruhn 1993; Colpaert 1999). *Laccaria* was isolated from stunted and healthy jack pine seedlings in 2007. *Sistotrema brinkmannii* was also isolated from stunted and healthy jack pine seedlings, and is typically classified as a wood decay fungi (Eriksson and others 1984); a follow-up study that was conducted on this fungus indicated it was neither a true mycorrhizae nor a pathogenic fungus (Potvin and others forthcoming).

None of the four fungal taxa were exclusively found on either the stunted or healthy seedlings. Although we observed some differences

Table 2. Acceptable foliar nutrient ranges for N, P, K, Ca, Mg, Mn and Zn (Youngberg 1985; Powers 1974) and values for healthy and stunted 1+0 seedlings analyzed in 2007. *P* values < 0.05 are statistically different.

	N	P	K	Ca	Mg	Mn	Zn
			----- % -----			----- ppm -----	
Acceptable	1.2 to 2.0	0.1 to 0.2	0.3 to 0.8	0.2 to 0.8	0.1 to 0.15	100 to 5000	10 to 125
Healthy 1+0	2.59	0.28	1.16	0.26	0.14	490	106
Stunted 1+0	1.00	0.12	0.60	0.70	0.19	385	57
***P* value**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 3. Total mycorrhizal root tip counts and average number of mycorrhizal root tips per 1-cm (0.4 in) root section for healthy and stunted 1+0, 2+0, and 3+0 jack pine seedlings in 2006. *P* values < 0.05 are statistically different.

	1+0 Seedlings			2+0 Seedlings			3+0 Seedlings		
	Healthy	Stunted	*P* value	Healthy	Stunted	*P* value	Healthy	Stunted	*P* value
Total mycorrhizal root tip count	1013	955	0.443	2172	1805	0.005	2058	1748	0.032
Average number of mycorrhizae/cm	3.38	3.18	0.443	7.24	6.02	0.005	6.86	5.83	0.032

in the frequency of them on stunted and healthy seedlings, these differences were inconsistent across age groups and years. We hypothesized differences would be present in the types of mycorrhizal fungi and between stunted and healthy seedlings and in the numbers of mycorrhizae on stunted and healthy seedlings. Our results do not support those hypotheses.

Work by Koll (2009) and Koll and others (2010) indicates that mosaic stunting observed at JW Toumey Nursery may reflect problems associated with excessive application of sawdust as a soil amendment.

Summary

It is traditionally thought that mosaic stunting in fumigated bareroot nursery beds occurs through these steps: 1) the biocide eliminates ectomycorrhizal fungi; 2) seedlings lacking ectomycorrhizae have poor nutrient uptake, especially P, and a reduced growth rate; 3) ectomycorrhizal fungi reinvade the nursery soil in a random fashion; 4) colonized seedlings have better growth than nearby non-inoculated seedlings, resulting in islands or pockets of remaining, stunted seedlings. Our results, however, indicate ectomycorrhizal fungi, or their quantities, are not a factor in jack pine seedling stunting at the JW Toumey Nursery, even though severely stunted seedlings were deficient in foliar N, P, and K. It may be that stunting at the nursery is a result of improper application of sawdust as a soil amendment.

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