Effect of altered nitrogen availability following accelerated succession of a forest: The role of tree species and size

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Abstract:

Many factors affect the amount of mycorrhizal colonization in a plant, such as soil fertility and plant species. We focus on the effect of accelerated succession, which changes nitrogen availability, on mycorrhizal colonization. Tree species and size were also of interest, as very few direct comparisons of species or size have been conducted. We measured colonization between sapling and canopy variants of *Acer rubrum* (red maple)*, Quercus rubra* (northern red oak)*,* and *Pinus strobus* (white pine) in a northern temperate forest in northern Michigan by analyzing fine roots using a well-developed gridline intersect method. These trees were located at two sites, one where succession was accelerated and one without accelerated succession that served as a control. Greater mycorrhizal colonization was measured in the forest undergoing accelerated succession, implying that several factors besides nutrient availability may be influencing colonization. Greater mycorrhizal colonization was also measured in the white pines, likely due to its coarser roots which rely more on mycorrhizal associations because of lower surface to volume area in the roots. It appears that both canopy and sapling individuals for each of the three study species have a similar reliance on mycorrhizal colonization, despite the different nutritional requirements between the two size classes.

1. Introduction:

Northern temperate forest trees, like most plants, rely on mycorrhizal associations for a significant portion of their nutrient uptake. Roughly 85% of all plant species are thought to have some form of mycorrhizal colonization (Kirk et. al, 2008). While simple diffusion of nutrients into the roots can provide much of the required materials needed for plant growth, diffusion through the roots is often not enough to sustain these plant species, especially in low nutrient environments. Increased absorption capabilities are provided by association with fungi, which aid absorption by increasing surface area, through the attached fungal hyphae, which are much smaller than even the finest root hairs (Kirk et. al, 2008). In return for the absorbed nutrients provided by the mycorrhizal fungi, the host plant provides

carbohydrates, such as glucose and sucrose, to the fungi. The net carbon balance for the plant must remain positive for it to remain advantageous, creating a nutrient exchange equilibrium required for the mutualism to persist.

The amount of mycorrhizal colonization present in a plant is thought to be governed by several factors, chief among these being the amount of nutrients available in the soil. The effect of nutrient availability on the amount of colonization has been well studied, with most findings showing an increase in colonization in nutrient poor soil and a decrease in nutrient rich soil (Treseder, 2004; Mosse & Phillips, 1971). If nutrients such as available nitrogen (N) (in the form of ammonium or nitrate), are more abundant in the soil, the plant no longer needs the fungi to aid in absorption. Because the plant needs carbohydrates as well, it is not economical for the plant to invest carbon sources into the mycorrhizal associations when N availability in the soil is high enough for the roots to directly uptake their entire nutrient requirements.

Nitrogen availability often limits that amount of carbon sequestration that is possible by forests (Keeney, 1980; Oren et al., 2001). Mycorrhizae may also serve as a significant form of soil carbon storage under increased CO_2 levels (Treseder & Allen, 2000). Carbon sequestration is becoming more important as atmospheric CO_2 levels continue to rise in the atmosphere due to anthropogenic causes. 15-30% of anthropogenic carbon is sequestered by carbon sinks on land, of which northern temperate forests are a big part (Myeni et al., 2001). The net primary production, and therefore amount of atmospheric carbon that is sequestered from the atmosphere, of these forests is limited primarily by N (Vitousek & Howarth, 1991). In sites of low fertility, or sites undergoing a disturbance that results in low nutrient levels, the nutrient deficit will negatively affect the amount of carbon sequestered by these forests.

Carbon sequestration and nitrogen availability change with forest disturbance and succession (Pastor, 1986; Vitousek et al., 1989). Succession is occurring throughout North American forests, especially in the Great Lakes region, where early-successional canopy species are dying and being replaced by later-successional species (Palik & Pregitzer, 1993). Given the long-lived nature of trees, succession is most often studied with chronosequences; however, our work is conducted in a recent, unique experiment designed to accelerate succession (Nave et al., 2011). To accelerate succession, our experimental site underwent ecosystem-level girdling that caused the death of the early successional species, *Betula papyrifera* (paper birch) and *Populus* spp. (aspen), thereby releasing the later successional species, predominantly *Acer rubrum* (red maple), *Pinus strobus* (white pine), and *Quercus rubra* (northern red oak). Girdling is also intended to mimic the effect that a pathogen or pest would

have when certain tree species are targeted. Such pathogenic disturbances are expected to increase in frequency with climate change, as pathogens find susceptible hosts in areas previously inaccessible due to unfavorable climate (Ayres & Lombardero, 2000). In this way, our study has implications for both climate induced changes to forest pests and pathogens as well as for succession-related changes to carbon and nitrogen cycling. The accelerated succession has increased soil N availability (Nave et al., 2011) and we expect for it to have an effect on mycorrhizal colonization as a result.

Colonization between tree species may be highly variable. This is attributed to different morphology, size, and nutrient requirements of each tree species. Colonization is also higher in tree species with coarser roots (Brundrett & Kendrick, 1989). Of the three species in our study, white pine tends to have the coarsest roots, so colonization amounts in these were expected to be highest. The type of mycorrhizae present might also affect colonization. Red maple is known to be colonized only by arbuscular mycorrhizal (AM) associations, while northern red oak and white pine form almost exclusively ectomycorrhizal (EM) associations in their roots. AM mycorrhizae have been found in many oak species (Watson et al., 1990), but they are predominantly colonized by EM and were treated as such.

Although much research has been done studying the effect of soil fertility on mycorrhizal colonization, little is known about the effect of tree size. An argument could be made for either canopy or sapling sizes having higher colonization amounts. The saplings may require greater colonization to obtain more nutrients needed for rapid growth. However, although growing less rapidly, the mere size of the canopy trees could demand high levels of overall colonization, since more nutrients and, as a result, more mycorrhizae are required to sustain the larger tree.

In this study, we test three hypotheses: 1) Mycorrhizal colonization decreases in trees located in the forest undergoing accelerated succession. 2) Mycorrhizal colonization differs by tree species and type of mycorrhizal associations (AM and EM). 3) Mycorrhizal colonization differs between sapling and canopy individuals of each species.

2. Materials and Methods:

Experimental Site:

The experimental site was located at the University of Michigan Biological Station (UMBS) in northern Michigan (45°35'N, 84°43'W) (Nave et al., 2011). The mean annual temperature at the UMBS site is 5.5^{o} C (1942-2003) and the mean annual precipitation is 817 mm including 294 cm of snowfall

(Nave et al., 2011). It is located on a high outwash plain and adjacent gently sloping moraine (Nave et al., 2011).

A wide-scale experiment was conducted at the UMBS to speed up the process of natural succession. The Forest Accelerated Succession ExperimenT (FASET) treatment consists of 39 hectares of land in which all of the *Populus* spp. and *Betula papyrifera* (approximately 6700 trees) were stem girdled to cause tree death in the spring of 2008. Stem girdling avoids root sprouting (Burns and Honkala, 1990) while at the same time eliminating targeted species, thereby enhancing succession rather than resetting it.

Root Harvesting:

Root samples were collected from 12 paired plots distributed across FASET and the control sites. The plots were chosen to cover a wide spectrum of above ground biomass and soil fertility. The paired plots were adjacent to each other but one received the girdled treatment and the other did not. Both canopy (> 8 cm DBH) and sapling (≤ 8 cm DBH) statures of each tree species were sampled. One canopy and one sapling of each species were sampled from each plot if they were present. Canopy pines were not present in two of the plot pairs and sapling pines were unavailable in one pair. To collect the roots, we used a pitchfork to soften a semicircle area of soil around the tree and traced roots from a target stem of each species until adequate samples were found. Our criteria for roots were any that were roughly smaller than 2 mm in diameter since these have been shown to be the sites where most of the nutrient absorption, and therefore mycorrhizal colonization, takes place (cite). The fine roots were then stored in labeled bags and kept under refrigeration to slow decomposition.

Root cleaning and clearing:

Soil and other debris were cleared from the fine roots by cleaning them in tubs of water and then separating the samples by mass (1-2 grams or less) and root order (3^{rd} order or less). The fine roots were cut into roughly 2-4 cm segments. AM cannot usually be seen under a dissecting microscope since most of the structures produced by AM are hidden by the tissues and pigments of the root (Vierheilig et al., 2005). The maple roots were the only ones that were predominantly colonized by AM, so clearing was only done to the maple samples. We found that EM roots (oak and pine) structures could be identified more easily without any clearing or staining. After cleaning, the maple samples were soaked in a 10% KOH water to volume solution to completely cover the roots (usually about 20mL) contained in glass containers and covered in tin foil. The glass beakers containing the roots were autoclaved on a liquids cycle of 121° C for one hour, which we found yielded the best results for the red maple.

AM root staining:

Once each maple sample was cleared, it was stained with aniline blue (cotton blue) to highlight the fungal hyphae (Brundrett et al. 1984). 0.03% water to volume dye was used. The samples were then autoclaved on a liquids cycle at 121° C for 15 minutes. Once the staining was complete the roots were destained in a solution of lactoglycerol for 1-2 days. Destaining makes the roots easier to analyze under a microscope by removing some of excess stain that may have attached to uncolonized root. Following destaining, the roots were evaluated for colonization.

Colonization analysis:

We used a modification of the grid line intersect method (Newman, 1966) to measure colonization in which roots are randomly dispersed in a square Petri plate with grid lines. We scanned the grid lines with a dissecting microscope to quantify intersections between grid lines and roots and designated these as either colonized or nonmycorrhizal. Different criteria were used for EM and AM roots, since AM roots were stained and EM were not. Since the stain attaches well to the fungi, a strong blue color was the main criteria for colonized intersection. For EM roots, morphology was the main indicator of colonization, with colonized roots appearing swollen and beaded at the tips. For both AM and EM roots, we counted 100 intersections each time and re-randomized the sample two additional times to improve accuracy for a total of 300 intersections per sample, as recommended by Giovanetti and Mosse (1980). The ratio of colonized intersections to total intersections gave us the proportion of the root length that was colonized by mycorrhizae, which was the primary measurement used in our data analysis.

Soil N and Basal Area:

No direct measurements of nitrogen were available at the time of this study, although ion exchange resin bags are expected to have these data in the near future. Since these data weren't available at the time of the study, basal area was used as a proxy measurement. DBH measurements were taken of both live and dead trees at each of the plots and basal area was calculated from these (m^2/ha). As the early successional species die off due to the girdling treatment, more nitrogen becomes freely available in the soil (Nave et al., 2011). By measuring dead basal area, it would give us an indication of the degree of girdling in each plot. Furthermore, since areas of high soil fertility seem to be correlated with total basal area, live and dead basal area combined might give a more accurate proxy of soil N.

Data analysis:

To determine if any statistically significant results were present we performed a three-way ANOVA test on the amount of colonization for each of the species (pine, maple, and oak), tree sizes

(sapling and canopy), and treatment (FASET and control). We used Fisher's LSD post hoc comparisons to identify significantly different groups. We then made linear regressions of colonization proportions depending on stand basal area and the DBH of the sampled tree in cm. Because our experimental site and samples were collected across a widely distributed field site (> 140 ha) and the gridline intersect counting method is most precise for roots grown under controlled conditions, we defined $p < .10$ to be significant for all categorical and continuous tests.

3. Results:

Treatment had a significant effect on the proportion of mycorrhizal colonization (t-test; $p < .10$), with the FASET treatment being slightly higher (Figure 1). The mean colonization of samples taken from the FASET plots was .17 (proportion of total root length) while it was .13 for the control (reference) plots.

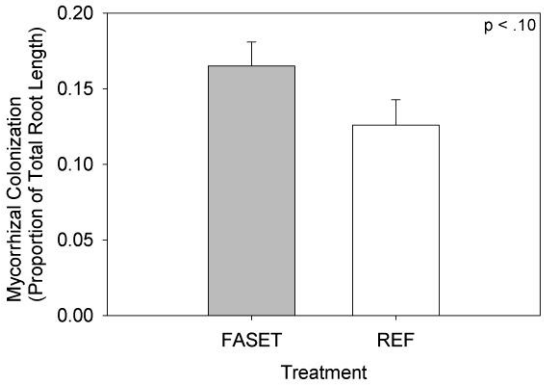

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Figure 1. Mycorrhizal colonization on root samples from FASET and control (REF) plots.

Pine was found to have had the greatest proportion of root mycorrhizal colonization among the three species (ANOVA; $p < .01$), with no significant difference between the maple and oak species ($p > .10$) (Figure 2). In terms of the proportion of the root length colonized, pine had an average colonization of .20 while oak had an average of .14 and maple had an average of .10.

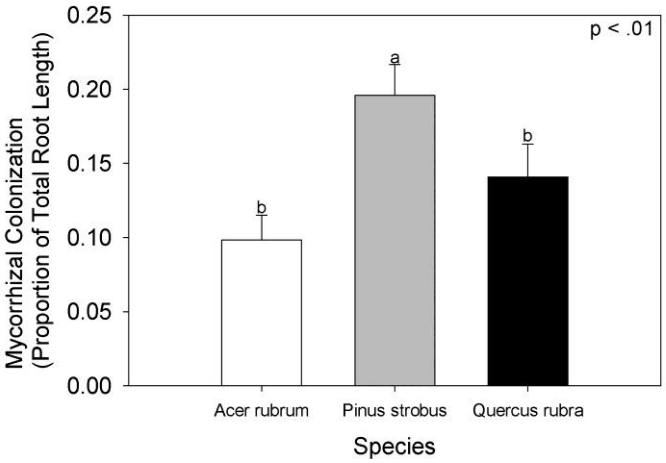


Figure 2. Proportion of mycorrhizal colonization within roots of Acer rubrum, Pinus strobus, and Quercus rubra.

Across all the study species, there was no significant difference in proportion colonized between the canopy and sapling size classes ($p > .10$) (Figure 3). Analysis of colonization among canopy and sapling individuals of each individual species yielded similar results, with no significant difference between maple, oak, or pine ($p > .10$).

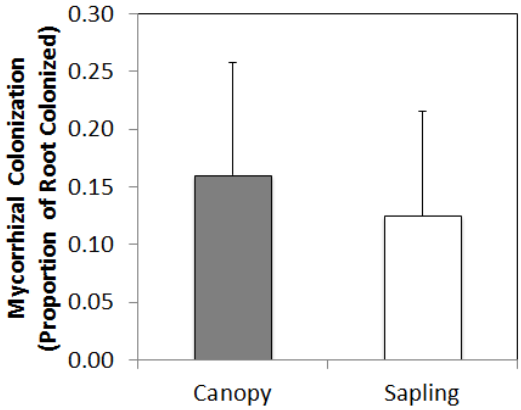
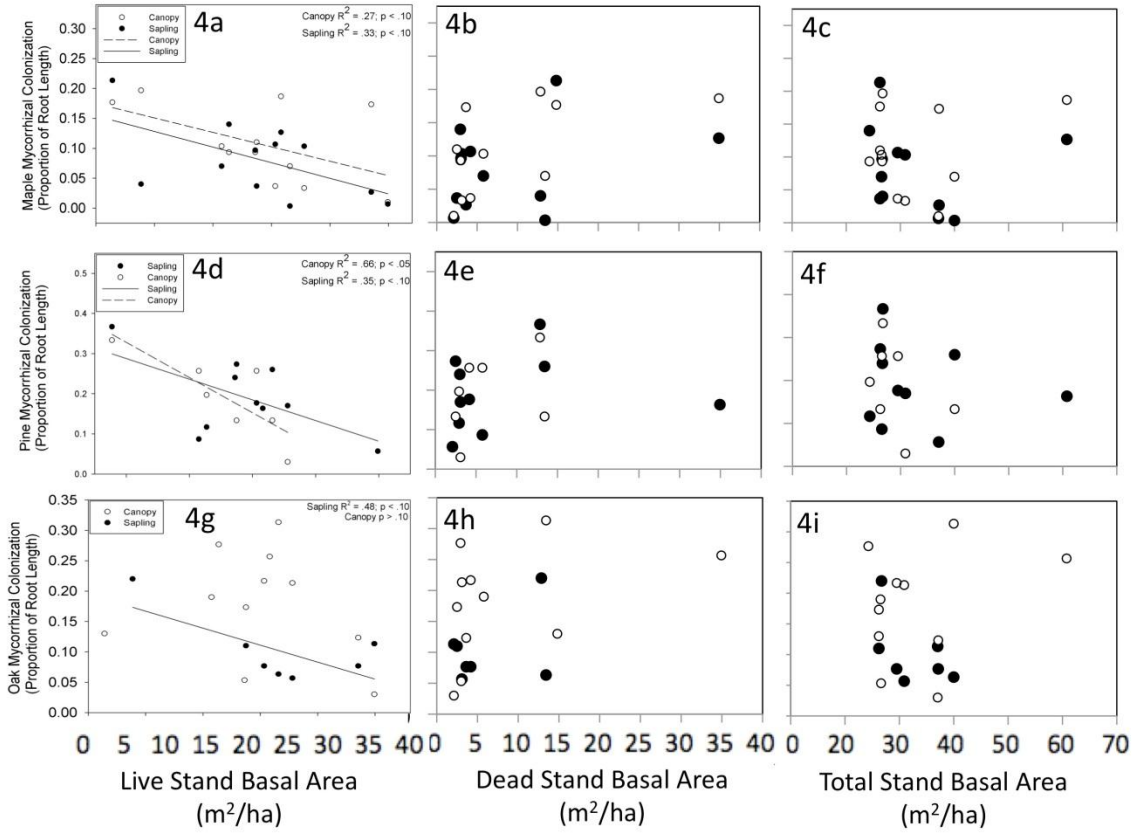


Figure 3. Combined average of the proportion of mycorrhizal colonization of the roots among all the species for canopy and sapling individuals.

No correlation was found between dead basal area or total basal area (live + dead) and colonization for any species and size ($p > .10$) (Figures 4b, 4e, 4h, 4c, 4f, 4i), while there was a negative correlation between colonization and both size classes of the pine and maple and the oak saplings (linear regression; $p < .10$) (Figures 4a, 4d, 4g).



Figures 4a-4i. Scatter plots of the proportion of mycorrhizal colonization in roots of maple, oak, and pine samples (rows 1, 2, and 3 respectively) vs. live, dead, and total basal area (columns 1, 2, and 3 respectively).

6. Discussion:

The roots harvested from the forest undergoing accelerated succession resulted in greater mycorrhizal colonization than the control forest. These results disagree with our original hypothesis that colonization amounts would be lower in the FASET site due to the increased nitrogen levels. The mechanism of increasing N in our study compared to previous work may be responsible for this discrepancy. Much of the evidence for decreasing colonization with increased N comes from fertilizer studies (Fransson et al., 1999; Wallenda & Kottke, 2008). These studies have larger N elevation effects and even N distribution. Our experiment involved altering nutrient levels indirectly by accelerating succession, which resulted in a much subtler change in soil fertility than the widespread application of

fertilizers. It is also expected that the nutrient increase caused by girdling the early successional trees would create nutrient-rich hotspots where the dying trees were located. These hotspots might cause a proliferation of roots (Crick and Grime, 1987) and fungal hyphae towards this spot, which may account for the observed increase in colonization.

We observed higher colonization amounts in the pine than in either of the maple or oak samples. Tree species with coarser roots tend to be more colonized than those with finer roots (Brundrett & Kendrick, 1989), so it was expected that pine, with coarser roots than either maple or oak, would have the most colonization. Naturally, coarser roots have less surface area to volume ratios and require the additional absorption capabilities provided by the fungi to meet their nutrient requirements. Other factors such as the abundance of species and type of mycorrhizae in the soil may have influenced colonization by species, but such characterization was beyond the scope of this project. For example, EM fungi are believed to have hyphal turnover that is much slower than AM hyphal turnover, due to the high chitin content of the fungal tissue of EM (Langley and Hungate, 2003). This may affect the rate at which trees colonized by different mycorrhizae reach nitrogen hot spots and, as a result, have an overall effect on root colonization by EM and AM tree species.

Mycorrhizal colonization was similar between the two size classes for each of our study species. To resolve the issue of the broad categorization of size classes, we also performed regression tests based on diameter at breast height (DBH) categorization. These assessments also showed that there is no correlation between the two, implying that while canopy and sapling variants of each species might have different nutritional requirements, they both seem to rely just as much on mycorrhizal associations. While this may be true for northern red oak, red maple, and white pine, other species may respond differently and further research is needed to conclude that rates of colonization do not differ between sapling and canopy variants of other tree species.

As N levels for our plots were not available at the time of the study, we thought basal area would serve as an accurate proxy for N. Dead basal area and the combined total basal area (dead and alive) logically seemed like good indicators of the amount of girdling, and therefore, nutrient levels in the soil. It was surprising that these basal area measurements did not correlate at all with colonization, suggesting that they either might not be good proxy measurements for N or that colonization is influenced by a variety of variables and not as simple as first imagined. It was interesting to note that almost all of the sapling and canopy variants of each species did show a correlation with stand basal area however. This reinforces the idea that colonization is not influenced primarily by soil nutrient

levels and instead influenced by a wide range of elements. Future N data for these plots will give a better indication of what is governing colonization amounts and how basal area plays in.

Concurrently with this study, another experiment was conducted by other researchers (Castillo, unpublished) on this project on the same root samples to measure the root content of fungal biomass. This companion test quantifies ergosterol, a component of fungal cell walls in many EM species. A linear regression analysis demonstrated that the colonization proportions of the oak and pine roots were correlated with root ergosterol contents (See Appendix, Figure 1A). The correlation, however weak it was, between the two methods assures us that both methods are accurately measuring mycorrhizal colonization in these roots. Because AM fungi often contain other sterols instead of ergosterol in their cell membranes (Schüssler et al., 2001), these ergosterol measurements and the resulting linear regression were only conducted for the white pine and northern red oak. While we could not verify colonization measurements for red maple roots, the gridline intersect method has been widely accepted for counting AM roots with a high level of success (Giovanetti & Mosse, 1980).

A couple of unwanted variables in the clearing and counting steps of our study may have negatively affected our results. Different counting methods were used between the AM and EM trees, since the red maple (AM) roots were cleared and stained while the northern red oak and white pine roots (EM) were not. Because of discrepancies in methods, our criteria for colonization were mainly based on morphology for the EM roots and color for the AM roots. The additional clearing step in the red maple may have also removed some of the mycorrhizae since the KOH solution inevitably created some unwanted disintegration of the root.

One study showed that many oak species, including *Quercus rubra,* were able to form AM associations as well as EM associations (Watson et al., 1990). We had trouble incorporating the clearing and staining methods for the northern red oak because most of our samples had been damaged too much by the clearing step, so we did not count any AM. This may have caused us to slightly underestimate the amount of colonization in the oak, but since this study involved greenhouse inoculation of the fungi, it is unlikely that field samples had any significant amount of AM associations.

Acknowledgements:

Thanks to everyone in Knute Nadelhoffer's lab, particularly Buck Castillo, Nicole Dear, Nick Van Dyke, and Clarisse Marie for helping to harvest and clean roots. Additional thanks to Buck for sharing the results of his study and spending additional time in each of the root harvesting, cleaning, and cutting steps.

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Appendix:

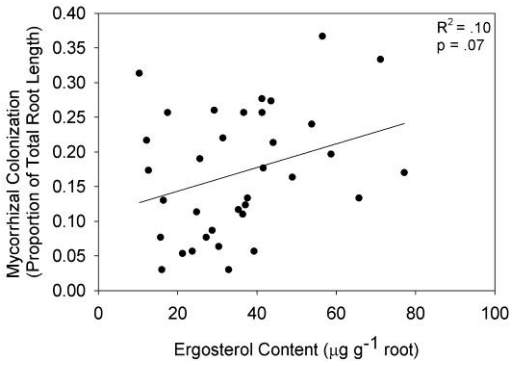

Figure 1A. Comparison of mycorrhizal colonization of pine and oak roots using microscopy with ergosterol content in the roots