Testing Predictions about Carbon- Nutrient Couplings in Forests:
A Dichotomy Between Mycorrhizal Root Types of Red Maple and Oak Plots at Harvard Forest

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Note from J. Hobbie: Jamie Bates says in the methods section (re: enzyme measures in roots) “The sample treatment involved separating the mycorrhizal fungi associated roots from the soil using gloves and forceps to gently scrape off adhering soil. The process took approximately 45-60 minutes per core. The mass of the roots were weighed and then placed in a drying oven at 50 degrees Celsius overnight.” I have checked with the T.A.’s and they stated that only a part of the mass of roots was dried overnight (this treatment would have destroyed enzyme activity).
Abstract:

Previous studies by Phillips and associates have predicted a specific dichotomy between carbon nutrient couplings based on tree species relationships with two types of mycorrhizal fungi. Phillips made measurements at two research forest at Moores Creek in Bloomington, Indiana with red maple trees and their associated with arbuscular mycorrhizal (AM) fungi and oak trees with their associated ectomycorrhizal (ECM) fungi (Phillips et al. 2013). In my study I used methods similar to those of Phillips (et al. 2013) to observe the ecology of mycorrhizae of AM and ECM tree species to compare the Phillips results from an Indiana forest with my results from the Harvard Forest in Petersham, MA.

The two types of fungi symbiotic with tree roots were observed by enzymatic analyses which tests for the potential activity of the enzymes phosphatase, cellulase, and chitinase. In contrast to the Phillips results, the phosphatase activity was greater in AM plots than in the ECM plots. Results of other tests did not show a difference between AM and ECM fungi. The framework for the nutrient economy of the AM and ECM forest that Phillips (et al. 2013) developed did not apply very well to the same type of forests in Massachusetts.

Key words/ phrases: mycorrhizae, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, hyphae, enzyme, amino acid, roots

Introduction:

Plants obtain nutrients from the soil via roots. As much as 90% of all plants have symbiotic relationships with mycorrhizal fungi (Pace 2003). The word mycorrhiza comes from myco meaning fungus and rhizae meaning root. These fungi aid the plant in obtaining water and nutrients from sources in the soil such as phosphorous, inorganic and organic nitrogen. The plant supplies the fungi with sugars; about 10-30% of photosynthetic carbon (Gadd 2007). My measurement focuses on carbon nutrient coupling through two types of fungi, arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi, relationships associated with two tree species; Acer rubrum, red maple, and Quercus rubra, oak trees, in Harvard Forest.
Richard Phillips and Edward Brzostek of the Department of Biology and Geography at Indiana University wrote a review paper that focuses on the effects of the combined fungi and roots on soil (Phillips et al. 2013).

There are three main components of the mycorrhizae including the root itself, the intraradical hyphae, or hyphae between the root cells or within the root cells, and extraradical hyphae, or fungal hyphae extending out from the root into soil (Utobo et al. 2011). Fungi take up free nutrients in the soil and also use extracellular enzymes to break down organic molecules and liberate nutrients for plants to take up. The hyphae often reach nutrient depletion zones which are inaccessible to roots. The measurements reported here tested whether the differences between these two types of forest trees, that is the AM and ECM associated species, noted by Phillips in the soils also existed in the mycorrhizae (roots plus fungi). The Phillips (et al. 2013) soil studies were conducted at Moores Creek forest in Bloomington, Indiana while the studies reported here occurred at Harvard Forest in Petersham, MA.

Each type of fungi is identified through physical and structural differences which allow them to reach specific nutrients within the rhizosphere. Arbuscular Mycorrhizal (AM) fungi, in association with red maple trees, enrich the amount of available phosphorus by extending hyphae below the roots (Phillips et al. 2013). The fungi are known to associate with 80% of plants including grasses, agricultural plants and trees: sassafras, red maple, and beech trees (Smith et al. 2008). Arbuscular mycorrhizal fungi are identified through structural appearances of which can only be viewed through a microscope. Characteristics of intracellular hyphae, or intraradical fungi, meaning hyphae within the plant cells and tree like structures within the root cells, or arbuscules (Image 1). Vesicles within the root cells are another indicator for AM fungi (Utobo et al. 2011). These fungi acquire inorganic nitrogen from decaying matter (Smith et al. 2011). It also appears that the plant roots in AM-dominated systems take up inorganic nitrogen, unlike the roots in ECM associations, although this point is debated.

Ectomycorrhizal (ECM) mycorrhiza have a thick fungal mantle. It consists of aggregated fungal hyphae around the root. Hyphae extend beyond the root and obtain nutrients at distances up to a half meter. These ECM fungi associate with shrubs and tree species including oak, pine, and beech (Phillips et al. 2013). ECM fungi have a greater carbon cost to the plant due to their ability to produce long-lived hyphae, some of which may live for more than a year, as well as their
access to nutrient pools which are untouched by AM fungi, obtain organic nitrogen and other nutrients (Allen et al. 2003). ECM produce extracellular enzymes, hydrolytic and oxidative, which degrade soil organic matter enabling them to mine soils for nitrogen contained in compounds such as chitin, proteins and phenol-protein complexes, and phosphate contained in organic compounds (Chalot et al. 1998).

The two types of fungi differ structurally and chemically, but both associate with trees and take up various forms of nitrogen. Here I wanted to use several methods to compare the roots to the soil based on Phillips’ review. Methods used by Phillips were for soil and so do not always translate into a good method for roots. The methods I used were enzymatic assays of roots with associated fungi and the quantification of hyphae by length. I also investigated the uptake of $^{14}$C leucine by roots from red maple and oak plots as well as a measure of total soil respiration.

**Methods:**

Over the course of the experiment there were two separate trips to the Harvard Forest in Petersham, MA. The first core samples were taken on November 6, 2013. The sampling day was cloudy, the air temperature was -1 degrees Celsius, and 2.5 cm of snow coated the ground. Samples were immediately put on ice to preserve in over the time from collection to analysis in Woods Hole, MA.

Three soil cores of five centimeters in diameter and 15 centimeters depth as well as the O horizon depth were obtained from six plots, three AM and three ECM. Each plot was about 4mx4m each. The AM and ECM plots contained 100% red maple trees and oak trees. Plot final selections were at random, but AM and ECM fungi plots were paired in order to have similar growth environments. The plots A, B, and C were linked by similar location (red maple plot 1 with oak plot 3, red maple plot 2, with oak plot 1, red maple plot 3 with oak plot 2). In each coupled plot all the trees were the same age as determined by the diameter of tree at breast height (DBH) (Table 1).
**Enzymatic Analyses of Mycorrhizae**

The enzymes were run within 4 days of sample collection and analyzed in duplicates of the three plots within each six, AM and ECM, plots. The root samples were tested for phosphatase, cellulase, and chitinase enzymes using substrates: MUF-phosphate which tests for the liberation of phosphorus from organic molecules; MUF-β-D-glucosidase (or –β-D-gluopyranoside) which tests for the break down of polymers of simple sugars; MUF-N-acetyl-β-D-glucosaminide (NAG) which tests for the break down of chitin and liberation of nitrogen from organic molecules (Phillips et al. 2013). Substrates were kept in the dark because MUF is sensitive to light (Vallino et al. 2013).

The sample treatment involved separating the mycorrhizal fungi associated roots from the soil using gloves and forceps to gently scrape off adhering soil. The process took approximately 45-60 minutes per core. The mass of the roots were weighed and then placed in a drying oven at 50 degrees Celsius overnight.

After drying the roots, the mass was recorded again, ground, and then made into a root slurry which were placed into 1.5mL falcon tubes. The slurry consisted of 0.2 grams root sample, which was ground with a mortar and pestle with 10 mL of DI water. Times were noted for the addition of 400 µL of enzyme substrates to each falcon tube. Then one milliliter of slurry and 4 mL of 200 mM glycine buffer with a pH of 10.5 was then added to each borosilicate tube. Initial and final samples, which were incubated for 2 hours, were then processed using a 10-AU Fluorometer (Turner Desgins; Sunnyvale, California). Data were analyzed for initial and final readings after 4 seconds of analysis for each tube.

**Soil Respiration**

The respiration data and core samples used for amino acid uptake and biomass were collected during the second visit to Harvard Forest on November 25, 2013. Approximately 1.25 cm of snow covered the forest floor. Leaf litter and snow were swept aside to clear an area for respiration chamber bases. These chamber bases, or rings, were staked in the ground so the bottom of the ring was partially in the soil to prevent air leaks. The chamber was then placed on the rings to record carbon dioxide flux into the chamber every 15 seconds using a LI-COR 6200.
A total of eight cores were collected on the second sampling trip and the same procedures followed. Cores from red maple plot 1 and Oak plot 3, plots that showed the most significant differences based on enzyme assay results, were taken last to limit the time out of the ground. If the root is out for too long it dies and becomes inactive. It was ideal to have active roots. The more alive the root the more likely the root would take up nutrients.

*Amino Acid Uptake of Roots*

Once back in Woods Hole, the two cores were sieved immediately, using 1 mm and 0.25 mm sieves, to gently clean the roots. Forceps and gloves were used again to scrape off soil. The root mass was recorded after being cut into one centimeter long pieces. In the radioactive lab I placed the roots into 2.0 mL microcentrifuge tubes containing 1.5 mL of cold, nonradioactive leucine concentrations 10, 25, 50, 100 µM solutions. I then pipetted 20 µL of hot, radioactive, $^{14}$C leucine to each microcentrifuge tube. Initial samples were killed, made inactive, with 0.1 mL of cold 100% TCA. The time was noted for time zero and approximately two hours later the final incubated samples were killed with the same amount of 100% TCA. The samples were centrifuged on high for 5 minutes.

I then used forceps to pull the roots from each sample. This was done to limit the risk of losing a root in the pouring process in removing radioactive waste from the microcentrifuge tube. The tube was dabbed dry using a Kimwipe. Samples were then washed twice with one milliliter of 50 mM KCl solution and the washing process was repeated by centrifuging and drying as before.

The roots were then crushed with a broken glass rod in the microcentrifuge tube. A plant tissue solubolizer, 0.5 mL Cellosolve (ethylene glycol monoethyl ether from Fischer Scientific), was added to the crushed roots. The samples were incubated for 24 hrs. Then 1.0 mL of ScintiSafe scintillation cocktail was added to the microcentrifuge tube, the tube placed in a glass scintillation vial, and the $^{14}$C incorporated was counted in a Beckman Coulter LS-6500 liquid scintillation counter.
Hyphal Length

A 350 watt blender (Hamilton Beach BlendMaster) was used to cut up and suspend 24 grams of roots and soil from each six cores in 500 mL of D.I. water. A 100 µL sample from the blended samples was mounted on a microscope slide and observed with a Widefield Zeiss AxioImager Z2 compound microscope at 100x magnification and transmitted light. About 20 field of view photographs were taken of each slide using ApoTome, a camera computer program. The hyphae were counted and the length of hyphae was noted with the ocular eye piece measurement. The diameter of the microscope field totaled 980 microns at magnification. Lengths observed were totaled and estimated as meters of hyphae per gram of soil.

Photographs for identification of whole roots were taken using a camera attached to the microscope. The roots were cleared, or bleached, by being boiled in hydrogen peroxide until the roots looked a pale yellow color.

Results:

Enzymatic Analysis of Mycorrhizae

I found the phosphatase enzymatic activity was greatest in red maple (AM) plots in contrast to oak (ECM) plots (Figure 1 & 2). The cellulase and chitinase activity did not show any significant difference between AM and ECM plots. Phillips and associates (2013) saw the opposite result where the activity of phosphatase was greatest in ECM plots.

Soil Respiration

I observed the soil respiration, measured with a Li-COR 6200, and the carbon dioxide flux did not show a difference between possible microbial activity in AM and ECM plots (Figure 3). The respiration in all plots was low. This could be due to late fall period, the cold temperatures, and presence of snow.
Amino Acid Uptake of Roots

The roots I sampled in AM and ECM plots showed no uptake of amino acids using 20 µL hot \(^{14}C\) leucine and additional 10, 25, 50, 100 µM cold leucine (Figure 4 & 5). The negative values representing the rate of leucine (amino acids) being taken up indicate that the roots were not active, or dead, during the incubation period of 2 hours.

Hyphal Length

Through observations under the Zeiss compound microscope I found no significant difference between AM and ECM length of hyphae (Figure 6). The sum of the hyphae across the three AM and ECM plots reflect that they have very similar lengths of 27-26 metres per gram of soil.

The AM and ECM fungi were identified under the microscope and photographed with the computer linked to the microscope (Image 1).

Discussion:

Enzymatic Analysis of Mycorrhizae

Arbuscular mycorrhizae are well known for enriching the phosphorus availability to plants, so I expected to see the most phosphatase enzyme activity in the red maple plots (Figure 1 & 2). The review paper by Phillips (et al. 2013) did not reach the same conclusions. Phillips and associates (2013) found through soil studies of the mycorrhizal-associated nutrient economy framework that ECM fungi had the most phosphatase activity 0.055 µmol\ h^{-1} g^{-1} in comparison with AM fungi 0.034 µmol\ h^{-1} g^{-1} (Phillips et al. 2013). Studies elsewhere do show that ectomycorrhizal fungi also break down protein complexes and P-bearing organic matter and it could be that there is much more mass of ECM fungi than AM Fungi. And it should be kept in mind that Phillips (et al. 2013) study measured enzymes in the total soil while my study measured the activity in roots isolated from soil. The ECM can weather minerals by releasing low-molecular weight organic chelators and hydrogen ions to make calcium and phosphorus more available (Van Breemen et al. 1998).
ECM are thought to have a higher carbon cost to associated plant species because the fungi can persist for a long time compared to the rapid turnover of AM fungi (Phillips et al. 2013). The fungi mine soils for N bearing compounds like chitin and protein complexes. From this I would have expected to see the highest chitinase and cellulase activity in the roots from oak plots. The measurements showed no enhancement of enzymatic activity in the red maple or the oak plots; unlike what Phillips saw. The data from Phillips showed ECM fungi had a NAGase enzyme activity of $0.014 \mu mol h^{-1} g^{-1}$ and AM had an activity of $0.006 \mu mol h^{-1} g^{-1}$ for the same enzyme.

**Soil Respiration**

The day we observed soil respiration was cold with snow present on the ground at Harvard Forest. This cold temperature and the onset of the winter season limited the carbon dioxide flux by inhibiting some microbial activity within the rhizosphere. The respiration I recorded was 90-125 mg $C m^{-2} d^{-1}$ which agrees with the range of 50-100 mg $C m^{-2} d^{-1}$ soil respiration of Harvard Forest in the winter of 1994-1995 (Figure 7; Boone et al. 1998). The lack of microbial and root activity may reflect the lack of nutrients being taken up by the fungi associated plants. Overall soil respiration showed no difference between AM and ECM plots.

**Amino Acid Uptake of Roots**

The methods for testing uptake of amino acids through the roots was replicated and edited from Melissa Campbell’s 2010 experiment. Campbell saw an increase of leucine amino acid uptake through oak roots in association with mycorrhizal fungi as concentrations increased (Campbell 2010). Campbell’s experiment measured leucine uptake at leucine concentrations of 0.1, 1, 10, 100, and 150 $\mu M$ with an incubation period of 30 minutes of oak roots. Campbell saw a range of 200-1400 $pmol/hr^{-1} g of dry root^{-1}$. This was very different from the negative leucine uptake seen in AM and ECM mycorrhizae. My oak roots show they are not taking up any amino acids so it is possible that their activity had already closed down the winter season (the leaves had already fallen).

Before conducting the experiment I ran a practice experiment using AM tree roots in Woods Hole and found a similar miniscule uptake of leucine using Campbell’s same method,
except for the using of a different tissue solubolizer and lengthened incubation time to two hours.
From this I changed the method to limit radioactive waste by adding a set amount of $^{14}\text{C}$-leucine to every experiment while adding various concentrations of non-labeled leucine. It should be noted that Campbell used ECM mycorrhizal roots while I used AM mycorrhizal roots so the experiments were not really comparable.

A few of factors could have resulted in the negative results: changing the methods by running samples in microcentrifuge tubes rather than just scint vials with a larger volume of tissue solubolizer, using cellosolve instead of ScintiGest as a tissue solubolizer, and the time duration between the roots being out of the ground for a longer period of time. The cellosolve could have had a slower degradation rate compared to ScintiGest. The roots from Harvard Forest were on ice for approximately three hours before being processed, unlike Campbell’s immediate experiment which was run within 40 minutes after collection.

The time of season in which the experiment was conducted is a reasonable hypothesis as to why the roots are not taking up amino acids. The lack of sugars being supplied to the fungi and frozen soils may result in this nutrient change. This brings up the question whether pines show similar results or if the remaining needles during the winter show a peak of activity via amino acid uptake. I explored this hypothesis in a pine root sample from MBL campus in Woods Hole, MA by examining uptake in 100 $\mu$M leucine. The sample was treated with the same methods as the oak and red maple roots, only it was immediately processed as Campbell’s experiment. The results were similar and miniscule like the red maple and oak samples from Harvard Forest. I suggest the methods be tested at warmer times of the year.

**Hyphal Length**

The hyphae lengths did not have a significant difference between arbuscular mycorrhizae and ectomycorrhizae. The approximate 27 metres of hyphae per gram of soil was low compared to other studies. Hyphal lengths are common to be in the units of kilometres per gram of soil (Marinucci et al 1983). Most researchers when working with fungi do not use one method exclusively because no method is superior (Rajapakse et al. 1994). This method has been used to quantify any differences in the amount of hyphae between AM and ECM fungi. For future studies there should be more replicate slides to count per sample to get a better average of hyphae per
sample and a dye should be used for the ease of counting. For viewing fungal structures within the root it is advised to clear and dye the roots with trypan blue or chlorazol black E to view the hyphae more easily (Utobo et al. 2011). For photographing the dye will make the image clearer because of the contrast between what is and is not fungi.

**Conclusion:**

The AM and ECM roots only showed a difference through the phosphatase enzyme assay where AM had more activity than ECM. This was different from Phillips’ results. The framework by Phillips which is based on sampling only the soils did not agree with results from samples of mycorrhizal roots in Harvard Forest. One difference between the comparisons is that the Harvard Forest plots were 4mx4m while the Indiana plots were 20mx20m (Phillips et al. 2013). Thus, both AM and ECM mycorrhizae may have been present in all samples. Another possibility is that the Harvard Forest plots had mixed leaf litter, that is both AM and ECM. The mixture of leaf litter could influence the fungi type and which nutrients are more abundant in soils. Finally, the time of year may have influenced the nutrient uptake and root activity results. The absence of photosynthesizing leaves greatly depleted or reduced entirely the supply of sugars to fungi. The time of year meant that the soils were cold and frozen at the surface which slowed down the enzymatic reactions.

It is not well known just how sensitive the mycorrhizae are to sampling delays- that is, the time-lag between field sampling and the beginning of laboratory experiments. The roots could have died between sampling and analyzing. If roots are going to be experimented upon I suggest processing samples as soon as possible for optimum results. Fungi associated with roots may also be sensitive and may lose activity. However, the fungi cannot be separated from the root for separate experiments as far as is now known. In conclusion, methods need more careful development and testing- in the case of this five week measurement period it is clear that we should have avoided those measurements that might be strongly effected by seasonal change. Two examples are the respiration and the incorporation of labeled amino acid. Both would be reduced by cold temperatures and by the cessation of the flow of sugars from the photosynthesizing tree to the fungi.
Acknowledgements

I want to present my unconditional gratitude for my mentor, John Hobbie, for teaching me everything I know about fungi and helping me out through this project. Yangtsho Gyaltshen for helping me sample in Harvard Forest and having the same interests. A thanks to Fiona Jevon for driving and for guiding Yangtsho and I to find our plots within Prospect Hill region of Harvard Forest. Thank you, Joe Vallino for assisting me in formulating 14C- Leucine methods. Jesse Sadowsky from UNH for answering some fungi- root method questions. Thank you Eddie Brzostek from the University of Indiana for answering questions about the review you collaborated with Richard Phillips in 2013.

Finally a Thank you to the teaching assistances: Alice Carter, Sarah Nelvan, Rich McHorney for being amazing as always and around for any questions I had. Thank you, Louie Kerr for microscope mountant. All the P.I.’s of SES for making this experience possible. Thanks to Lauren Wind, Kelsey Geosslin, and Sarah Erskin/all of the students of SES for the moral support and companionship.
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Tables & Figures:

Table 1: The diameter of the tree at breast height across the three plots of AM and ECM plots.

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**Figure 1**: The average of duplicated enzyme assays of phosphatase, cellulase, and chitinase of red maple roots in plots A, B, and C.
Figure 2: The average of duplicated replicates of enzyme activity of phosphatase, cellulase, and chitinase within roots from oak plots A, B, and C.
Figure 3: The average of three soil respiration analyses in the three plots of both AM and ECM.
Figure 4: The amino acid uptake of red maple plot 3 (plot A) after an incubation of 2 hours using hot, 14C-Leucine, and cold leucine of concentrations 10, 25, 50, and 100 µM.
Figure 5: The amino acid uptake in pico moles of leucine/hour/gram of root from oak plot 3 (plot A) in 10, 25, 50, and 100 micro-molar concentrations.
Figure 6: The length (meters) of hyphae per sample (24 grams of roots and soil) of three AM and ECM plots in Harvard Forest.
Figure 7: Soil CO2 efflux per treatment for Harvard Forest litter-manipulation plots:
Measurements were made over a one-year period from 16 June (Julian day 167) 1994 to 14 June
(Julian day 165) 1995. Values are treatment means (n = 3 except for control, for which n = 6) of
two measurements (early morning and late afternoon) within the same day. The means of
coefficients of variation over the sampling period ranged from 10% to 15% by treatment. CO2-C,
carbon in the form of carbon dioxide (Boone 1998).
Image 1: A) Arbuscular Mycorrhizal fungi viewed under Zeiss compound microscope at 100x magnification showing intracellular hyphae and arbuscules. B) Ectomycorrhizal fungi showing thick mantle of mycelium around the root. C) ECM root showing root tip with removed mantle and hyphae pointing out at the root tip. D) ECM root with hyphae sticking out of the root tip.