

THE EFFECTS OF NITROGEN ADDITION ON MYCORRHIZAE: ANALYSIS OF STABLE NITROGEN ISOTOPES

JORDAN M. KRAMER

Abstract: Under natural conditions, leaves of Pitch Pine *Pinus rigida*, Black Huckleberry *Gaylussacia baccata*, and Black Oak *Quercus velutina* had $\delta^{15}\text{N}$ signatures that were depleted compared to soils, indicating the use of mycorrhizae for nitrogen uptake. Mycorrhizal fungi had enriched $\delta^{15}\text{N}$ values. With the addition of nitrogen rich wastewater, oak and huckleberry $\delta^{15}\text{N}$ values showed continued reliance on mycorrhizae. Pine $\delta^{15}\text{N}$ values were enriched with an average value equal to the wastewater $\delta^{15}\text{N}$ value of +9.0‰. This suggests that pines were able to use the dissolved inorganic nitrogen in the wastewater instead the fungi-supplied nitrogen. Mycorrhizal fungi were less enriched in wastewater fertilized plots and total hyphal abundance decreased.

Key Words: *Mycorrhizae; Stable isotope; Nitrogen addition; Fractionation; Pinus rigida; Gaylussacia baccata; Quercus velutina*

INTRODUCTION

It has been shown that many plants depend on fungi for some portion of their nutrient uptake. In these mutual relationships, known as mycorrhizae, fungi provide nitrogen in the form of amino acids to plants (Hobbie and Colpaert 2003). Plants in return provide sugars to the fungi. The importance and magnitude of this exchange is unknown. Understanding this exchange would provide a greater understanding of forest nutrient cycling and the nutritive strategies of both plants and mycorrhizal fungi.

It is crucial to view mycorrhizal relationships under different conditions to see if the concentration of nutrients and the forms they are available in affect fungi-plant dynamics. Fungi can easily draw nutrients from organic and inorganic pools while plants are limited to inorganic nutrients (and very small simple organic molecules). If plants are given enough readily available nitrogen will they still invest energy in the form of sugars to mycorrhizae? Can mycorrhizae be shut out or shut off, or are they obligate?

This study follows the approach of Hobbie et al. 1999. In that study it was demonstrated that nitrogen transfer through mycorrhizae results in a fractionation where fungi become isotopically enriched while plants become isotopically depleted. When fungi build amino acids for transfer to plants, ^{14}N is preferentially used. A higher proportion of the heavier ^{15}N stays in the fungus (Figure 1). We can use the amount of ^{15}N depletion in trees compared to soils to see how much nitrogen is derived from fungi. We can also compare the amount of fungal ^{15}N enrichment to soil to see how much nitrogen is given up to plants. The equation below can be used to find enrichment or depletion of a sample compared to air.

$$\delta^{15}\text{N} = \left[\left(\frac{^{15}\text{N}_{\text{sample}}}{^{14}\text{N}_{\text{sample}}} \right) / \left(\frac{^{15}\text{N}_{\text{air}}}{^{14}\text{N}_{\text{air}}} \right) - 1 \right] \times 10^3$$

In the measurements reported here, I used stable isotope analysis to establish the relative importance of mycorrhizae to nitrogen uptake in three plant species; black oak (*Quercus velutina*), black huckleberry (*Gaylussacia baccata*), and pitch pine (*Pinus rigida*) and to investigate the mycorrhizal properties of the fungi.

I examined these relationships under two different sets of conditions at the Falmouth, MA Wastewater Treatment Forest (Figure 2). One field site is a pitch pine dominated forest that has been sprayed with nitrogen rich wastewater for the past 17 years (with an average deposition of 549 Kg N/ha/yr) (Thoms et al. 2003). Within this fertilized site are two sub plots F1 and F2. The other site is a similar adjacent pitch pine forest that has not been sprayed. This is the control site with two sampling areas C1 and C2. The sub plots were 10x40m. I hope to see if the influx of dissolved inorganic nitrogen (DIN), which plants are able to incorporate without mycorrhizae, changes the nature or prevalence of the mycorrhizal mutualism. I will also examine the effect of the nitrogen addition on the hyphal density of the organic soil.

METHODS

Mushrooms were collected between Oct 1 and Nov 15, 2003, and identified to at least genus. The 11 suspected mycorrhizal species (genus was listed as mycorrhizal in Hobbie et al. 2001, the Canadian Department of Natural Resources, or Agerer 2002) were then dried at 50°C and ground to a fine powder using a Wig-L-Bug Amalgamator (Crescent Dental Manufacturing Co. Lyon, IL). A known saprotroph (non-mycorrhizal fungi) *Mycena sp.* was also dried and ground as a comparison. Green leaves (20+/site/species) were collected in early October and dried at 50°C. Dried leaves were ground with a Wiley Mill. Soils (3 samples/site) were collected from the organic layer, dried at 50°C, ground with mortar and pestle, and homogenized. Organic soils had the highest root and hyphal densities and were used as the only soil samples due to cost constraints for stable isotope analysis. All dried ground samples were weighed to 0.0001mg then were given to the MBL stable isotope laboratory for %N and ¹⁵N measurements.

DIN was extracted from wet soil with KCl and gravity filtration (5g soil +10mL 1M KCl shaken for 1 hour and filtered through a Whatman #42 filter) (Method adapted from SSSA 1994). Ammonium was measured using colorimetric analysis on a Shimadzu 1601 spectrophotometer (Shimadzu Scientific Instruments Inc. Columbia, MD) (Method adapted from Strickland et al 1972). Nitrate samples were filtered again through a Whatman 0.45um syringe filter. Samples from control plots were diluted 2:1 and fertilized plot samples were diluted 20:1. These samples were run on a run on a spectrophotometric auto analyzer (Lachat Quick Chem 8000). Total N loading and ¹⁵N data for spray water were taken from Jordan et al. 1997 and Thoms et al. 2003.

For hyphal counts, 2 g of wet soil were diluted and stored in 10 ml 5% gluteraldehyde 95% phosphate buffered saline (PBS). Two and a half ml of this slurry was added to 49 ml of PBS. These samples were each ground on “liquefy” in a standard blender for 1 minute. Half of a milliliter of each blended diluted sample was stained for two minutes with a fungi specific dye Fungifluor (0.05% Cellufluor, Polysciences Inc.) and were filtered through 0.8 um Poretics white membrane filters (Osmonics). Counts were done under 100X magnification with an excitation wavelength of 460 nm on a Zeiss Axioscope microscope. Soil samples from each sub site (C1, C2, F1 and F2) were taken at each site in triplicate and 10 adjacent 100 um² fields were counted on each slide.

ASSUMPTIONS

For interpretation of data, some assumptions needed to be made. The $\delta^{15}\text{N}$ of bulk soil was considered to equal the total $\delta^{15}\text{N}$ of nitrogen available to fungi (a composite of DIN and organic N). It was also assumed that there was no major fractionation during fungal uptake (or plant uptake under nitrogen limitation). I therefore assume that any difference in plant or fungal

$\delta^{15}\text{N}$ from bulk soil is due to fractionation in the fungi before transfer to the plant. I also assumed that the $\delta^{15}\text{N}$ of saprotrophic fungi was representative of the organic matter pool and that the $\delta^{15}\text{N}$ of plants without (or with few) mycorrhizae would be representative of the DIN pool.

RESULTS

Site Soil Data

Control plots had much lower DIN than fertilized plots. NH_4^+ was found in higher concentrations than NO_3^- in all plots (Figure 3 and Table 1). Fertilized plot 2 (F2) had significantly higher DIN than F1. Bulk soil %N was between 1% and 1.6% in all sites (Figure 4). N loading due to fertilization was 459 Kg/ha/yr. Average bulk soil $\delta^{15}\text{N}$ was -0.2‰ for control plots and +5.2‰ for fertilized plots (Figures 5 & 6). Spray water was on average +9.0‰ compared to air (Thoms et al. 2003). In the inorganic nutrient pool (from KCL extractions) nitrogen was limiting in all sites (Table 2).

PO_4^{3-} concentrations were similar across all sites and ranged from 1.71 mg P/kg soil to 2.32 mg/kg. N:P ratios showed P limitation in all bulk soil sampled. This pattern may change temporally with spray (excess nitrogen from spraying is found in runoff and ground water) (Thoms, et al. 2003).

Plant $\delta^{15}\text{N}$

All oak leaves were depleted compared to bulk soil values (Figures 5 & 6, Table 3). Oaks in Fertilized 1 were the most depleted at -8.2‰ below bulk soil. Control 2 oaks were -3.7‰. Control 1 and Fertilized 2 were only depleted by -0.1‰ and -0.4‰ respectively. All huckleberry leaves were depleted as well. The greatest depletion was seen in F1 (-7.9‰) then F2 (-4.2‰), C1 (-2.8‰) and C1 (-0.7‰). Pine needles in control plots were depleted compared to soil (-3.4‰ for C1 and -0.3‰ in C2). In fertilized plots pine needles were enriched compared to bulk soil (+2.3‰ for F1 and +5.2‰ for F2).

Fungal $\delta^{15}\text{N}$

The *Boletus* samples (found only in control plots) were the most enriched at +11.3‰ in C1 and +13‰ in C2 compared to soil (Table 4). *Russula* was enriched by +9.4‰ in C2 and +5.7‰ in F2. *Laccaria* (found only in control plots) was enriched by +7.8‰ in C1 and +7.6‰ in C2. *Sclerodermas* (found only in F1) was enriched by +6.7‰. *Mycena*, a reported saprotroph, was enriched by +3.5‰. Two species of *Claveria* [*cinerea* (+3.8‰) and *delphus* (+4.0‰)] and *Lycoperdon* (+4.5‰) had similar enrichment values. *Amanita* found in F1 were enriched by +1.4‰.

Hyphal Abundance

Hyphal volume was highest in C2 (26998 $\mu\text{m}^3/\text{mg}$ soil), then C1 (22979 $\mu\text{m}^3/\text{mg}$), F1 (20953 $\mu\text{m}^3/\text{mg}$), and F2 (16467 $\mu\text{m}^3/\text{mg}$) (Figure 7).

DISCUSSION

Control plot $\delta^{15}\text{N}$

In the control plots, *Boletus*, *Russula*, and *Laccaria* appeared to be mycorrhizal. *Boletus* appeared to have the strongest mycorrhizal properties. Samples in both control plots were the most enriched, but also varied by 1.7‰. *Russula* samples were also strongly enriched. Three species from genus' that had been listed as possibly mycorrhizal were significantly less enriched

and were grouped closely with *Mycena*, a reported pure saprotroph. These species appear to be acting as saprotrophs in the system, and their $\delta^{15}\text{N}$ signature gives us an estimate of the organic soil nitrogen pool available to fungi. Enrichment values for mycorrhizal fungi were higher than similar measurements taken in arctic studies (Hobbie et al 1999, Lilleskov et al. 2002)

All sampled plants were depleted compared to soils. Depletion varied between C1 and C2 and between species from -0.1‰ to -3.7‰ . The least depleted sample was equal to the highest amount of depletion seen in culture by Hoberg et al. 1999. The range of depletion was within the range of values found in culture by Hobbie and Colpaert 2003. Both studies observed *Pinus sylvestris* in pots. $\delta^{15}\text{N}$ of plants was also consistent with value from field research at Glacier Bay, Alaska (Hobbie et al. 1999).

The averages for plants showed a small range of only 0.3‰ . On average pines were the most depleted, and therefore the most influenced by mycorrhizae. Oaks and huckleberry were the next most depleted..

Changes in the system with N addition

Leaves of all huckleberry and F2 oak were more depleted compared to source soil in the fertilized. This suggests that more nitrogen is derived from fungal sources than in natural conditions. The F1 oaks are similarly depleted compared to control oaks.

Pines were enriched compared to bulk soil. The source of this $\delta^{15}\text{N}$ appears to be the enriched spray-water. Average pine $\delta^{15}\text{N}$ and spray-water $\delta^{15}\text{N}$ have the same enrichment ($+9.0\text{‰}$). DIN from spray-water seems to be a much more important source of nitrogen to pines than mycorrhizae.

Oaks and huckleberry could remain strongly mycorrhizal during fertilization for a number of reasons ranging from dependence on fungal uptake for nutrients not provided in spray-water, to close evolutionary ties to fungi that do not permit complete non-mycorrhizal uptake. New root growth may also be more costly than maintenance of existing mycorrhizal roots (Tingey et al 1995).

By plotting DIN against the change in $\delta^{15}\text{N}$ from soil (Figure 8), we can see how plants respond to the fertilization. A positive slope shows less dependence on mycorrhizae while a negative slope show increased use of mycorrhizae. Oak and especially pine lose dependence on mycorrhizae while huckleberries receive more nitrogen from mycorrhizae with added DIN.

Fungi are less enriched in fertilized plots, showing a reduction in mycorrhizal activity with increased DIN. *Schlerodermas* had the strongest mycorrhizal signal. *Russula* was next, but was far less enriched than the sample from C2. The *Amanita* sample was only slightly enriched compared to soil.

Hyphal density is lower in fertilized plots. This measurement represents the general fungal pool of both saprotrophs and mycorrhizal fungi. In other studies (reviewed in Wallenda et al.) nitrogen addition slightly reduced hyphal density and root tip colonization.

Variation between plots could be caused by spatial variation in nitrogen forms or differences in species composition. Different fungi might have different nitrogen sources or might be specific to a certain host plant. The jump in pine $\delta^{15}\text{N}$ could be explained by the loss of a pine-specific mycorrhizal fungi. Species diversity of fungal fruiting bodies was 66% lower in fertilized plots during the sampling window. Wallenda and Kottke (1997) reviewed the response of mycorrhizal fungi to nitrogen fertilization. They found that long-term nitrogen addition had the strongest negative effects were on fruiting bodies and conifer-specific fungi.

CONCLUSION

From the data in this experiment, the relative importance of mycorrhizae to plants and fungi can be examined by looking at the extent of enrichment or depletion. We can see, for instance, that the *Boletus* in this forest is more mycorrhizal than *Laccaria* and pine is more mycorrhizal than oak. We do not know, however, the amount of nitrogen that passed from fungi to plant and the amount of nitrogen in the plant derived from fungi.

Hobbie and Hobbie (2003) have developed a model that uses the $\delta^{15}\text{N}$ of plants, fungi and available soil nitrogen to calculate the percent of plant nitrogen derived from fungal sources and the $\delta^{15}\text{N}$ value of the nitrogen passed from the fungi to the plant. These values change with different amounts of fungal nitrogen retention. If we put reasonable parameters on the percent of N derived from fungi and $\delta^{15}\text{N}$ supplied by fungi, we can develop a range of nitrogen retention for the fungi. Using the equations below from Hobbie and Hobbie 2003, I calculated the range of possible fungal nitrogen retentions for pines in the control plots.

$$(1) 100\% (\delta^{15}\text{N}_{\text{available}}) = (\text{fraction stays in hyphae})(\delta^{15}\text{N}_{\text{hyphae}}) + (\text{fraction transferred to plant})(\delta^{15}\text{N}_{\text{transfer}})$$

$$(2) 100\% (\delta^{15}\text{N}_{\text{plant}}) = x (\delta^{15}\text{N}_{\text{transfer}}) + (1-x) (\delta^{15}\text{N}_{\text{available}})$$

For my sample calculations I used average mycorrhizal and pine $\delta^{15}\text{N}$ with the $\delta^{15}\text{N}$ of the organic soil pool as the source nitrogen. The fungal contribution to plant nitrogen could be up to 100%. I assumed that fractionation had a limit around 10‰. With these parameters the minimum amount of plant nitrogen derived from mycorrhizae was 58% (Table 4).

The range determined by these calculations shows that nitrogen transfer by mycorrhizae is substantial in the control system. With this model over half of the nitrogen in pines, oaks, and huckleberries was derived from fungi. We also see that mycorrhizal fungi can only transfer about half of their nitrogen to plants in this system. In the fertilized system the mycorrhizal dynamics change. Pines receive less than 1% of their nitrogen from mycorrhizae while oaks and huckleberries must receive 75% and 92% from mycorrhizae respectively.

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TABLES AND FIGURES

Table 1: Nitrogen loading and DIN values

Table 2: N:P ratios of soil extracts

Table 3: Enrichment and depletion relative to soil

Table 4: Sample calculations for nitrogen balance

Figure 1: ^{15}N Fractionation model from Hobbie et al. 1999

Figure 2: Map of Falmouth, MA Wastewater Treatment Forest from Jordan et al. 1997

Figure 3: Extractable DIN in each plot

Figure 4: Bulk soil % nitrogen

Figure 5: $\delta^{15}\text{N}$ of leaves, soils, and fungal fruiting bodies

Figure 6: Average $\delta^{15}\text{N}$ for leaves, soils, and fungal fruiting bodies

Figure 7: Hyphal volume in each plot

Figure 8: Changes in $\delta^{15}\text{N}$ with nitrogen addition

Table 1

	Kramer 2003						Lilleskov, Hobbie and Fahey '01		
	PC1	PC2	PC Ave	PF1	PF2	PF Ave	high	med	low
Estimated Additional Loading Kg/ha/yr	XXXXX			549.04			14.64	6.78	1.62
mg/kg NO3	0.01809	0.030712	0.024401	0.45	2.17	1.31	49.70	26.90	1.81
mg/kg NH4	0.20	0.18	0.19	0.55	2.54	1.55	134.00	104.00	29.00
Bulk Soil %N	1.17	1.35	1.26	1.56	1.05	1.30	XXXXX	XXXXX	XXXXX

Table 2

Plot	N:P
C1	0.33
C2	0.27
F1	1.32
F2	6.33

Table 3

C1			
Leaves	15N Relative to Soil	Fungi	15N Relative to Soil
Oak	-0.4	Boletus	11.3
Oak Rep.	-0.1	Clavaria C	3.8
Pine	-3.4	Laccaria	7.8
Huck	-0.7	Lycoperdon	4.5

C2			
Leaves	15N Relative to Soil	Fungi	15N Relative to Soil
Oak	-3.7	Boletus	13.0
Pine	-0.3	Clavaria D	4.0
Huck	-2.8	Laccaria	7.6
		Russula	9.4
		Mycena	3.5

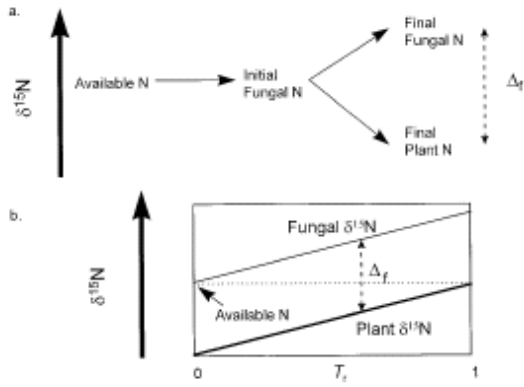
F1					
Leaves	15N Relative to Soil	15N Relative to Spray	Fungi	15N Relative to Soil	15N Relative to Spray
Oak	-8.2	-11.9	Amanita	1.4	-2.3
Pine	2.3	-1.4	Sclerodermas	6.7	3.0
Huck	-7.9	-11.6			

F2					
Leaves	15N Relative to Soil	15N Relative to Spray	Fungi	15N Relative to Soil	15N Relative to Spray
Oak	-0.1	-4.0	Russula	5.7	1.8
Pine	5.2	1.4			
Huck	-4.2	-8.0			

Table 4

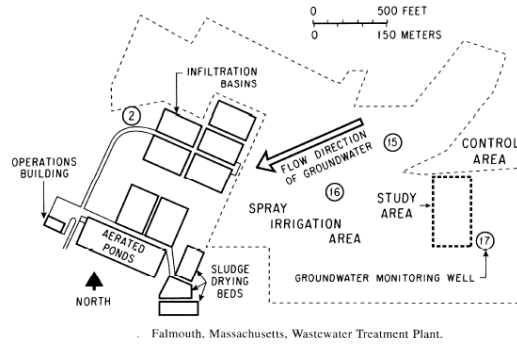
	<--max fractionation			max fungal N in plant-->	
fraction N that stays in hyphae	0.63	0.60	0.57	0.54	0.50
fraction N that is transferred to plant	0.37	0.40	0.43	0.46	0.50
del15N transferred to plant	-6.15	-4.95	-3.92	-3.03	-2.00
total fractionation for allocation to plant	-10.05	-8.85	-7.82	-6.93	-5.90
% N in plant from hyphae	58%	66%	74%	84%	98%

Figure 1 from Hobbie et al. 1999



Hypothesized patterns of N movement and isotopic fractionation in the plant-fungal-soil system, assuming $f=1$. a Reaction schematic. b Variations in plant and fungal $\delta^{15}\text{N}$ as T_t changes

Figure 2 from Jordan et al. 1997



Falmouth, Massachusetts, Wastewater Treatment Plant.

Figure 3

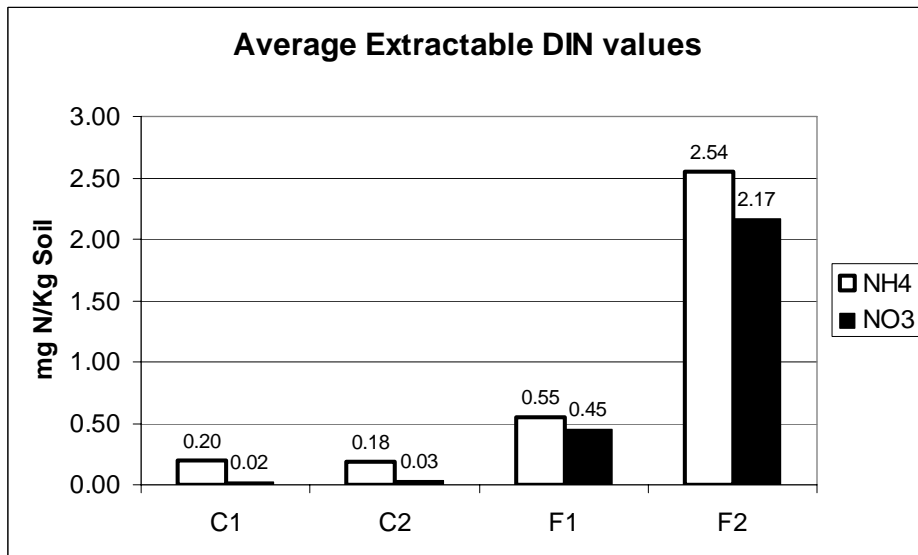


Figure 4

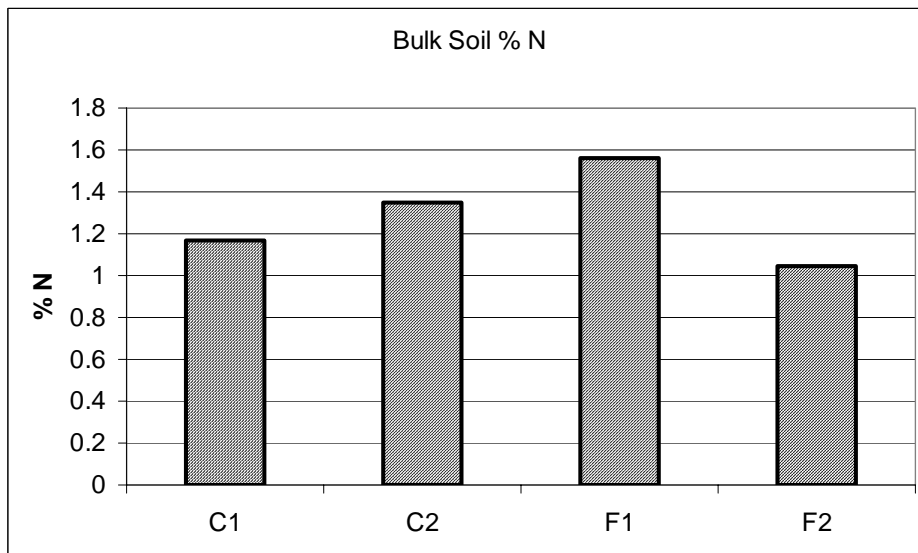


Figure 5

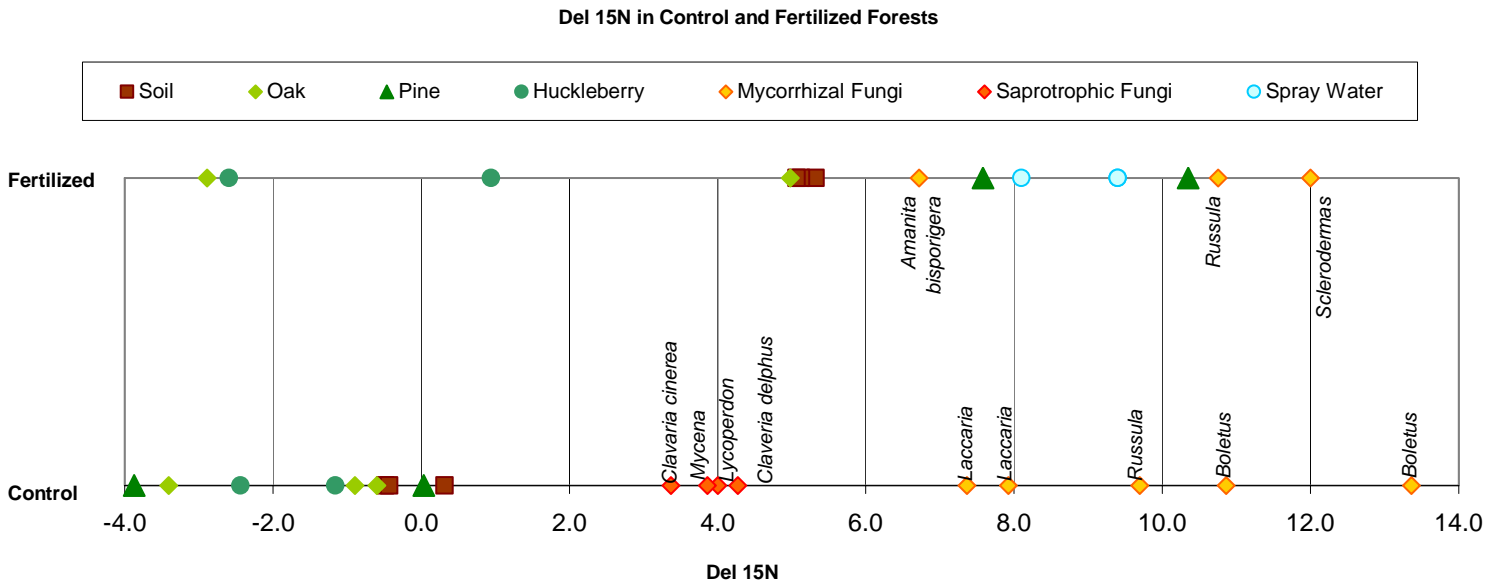


Figure 6

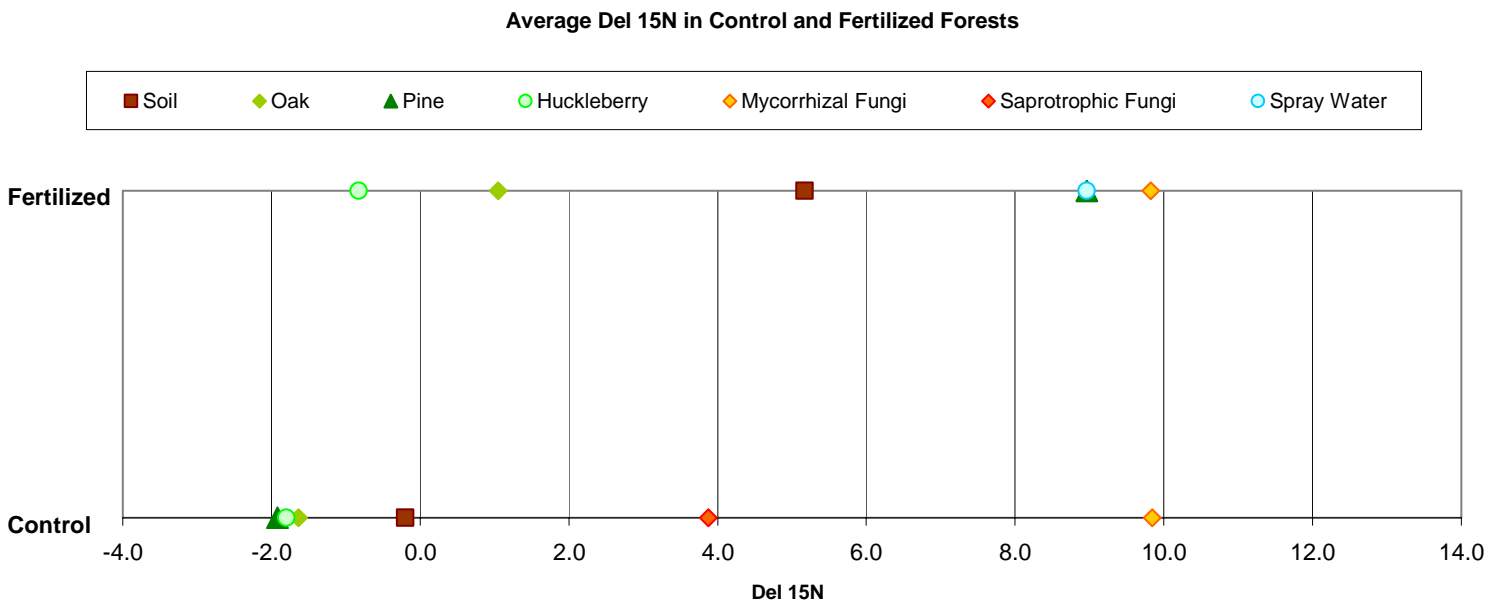


Figure 7

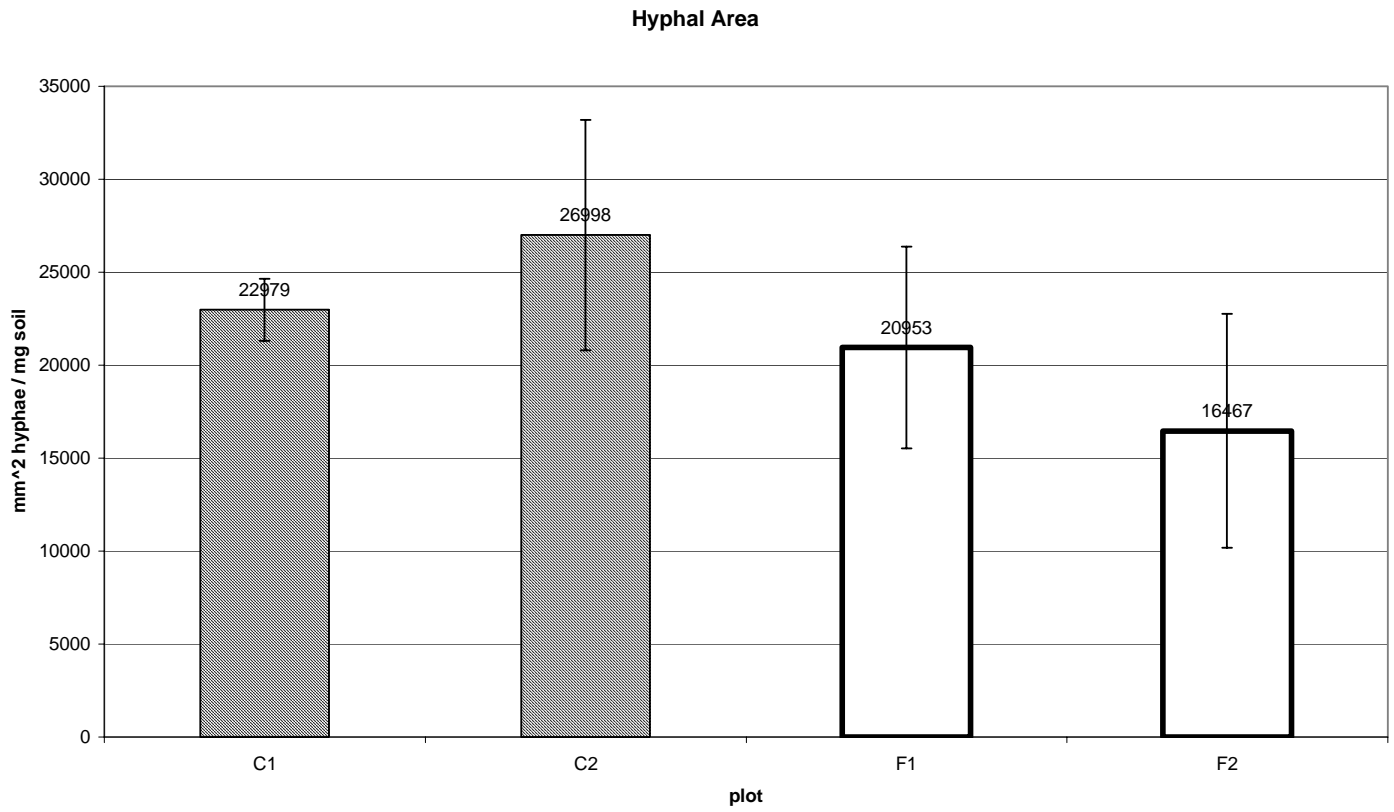


Figure 8

