THE VERTICAL DISTRIBUTION OF ERICOID, ECTO, AND ARBUSCULAR MYCORRHIZAE IN A MIXED SPECIES FOREST SOIL: CORRELATIONS WITH SOIL N AND P DISTRIBUTION AND AFFECT ON ROOT TOPOLOGY

by

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(Under the direction of Ronald L. Hendrick)

ABSTRACT

Understanding the factors affecting the spatial distribution of mycorrhizal fungi in soils is critical to understanding their impacts on forest communities. I investigated the vertical distribution, of ericoid (ERM) ecto- (ECM), arbuscular (AM), and mycorrhizal fungi in a soil at Coweeta Hydrologic Lab, Otto, N.C., and attempted to determine if this spatial distribution was correlated with N and P fraction distribution. The soil studied maintained host plants for all three mycorrhizal fungi, *Rhododendron maximum* (ERM), *Tsuga canadensis* (ECM), *Liriodendron tulipifera* (AM).. We utilized DNA sequencing to determine mycorrhizal fungal distribution. Sequences were identified using BLAST searches and by utilizing Maximum-Likelihood analysis. The correlation between mycorrhizal fungal distribution and N and P distribution was tested using Principal Component Analysis. The results indicate that the three mycorrhizal types are differentially distributed throughout soil horizons. ERM fungi occurred predominately in O horizons and AM fungi occurred mainly in B horizons. The majority of ECM fungi were located within the A horizon but were found in the O and B as well. ERM fungi were positively correlated with high concentrations of inorganic N and organic N and P. AM fungi were negatively correlated with inorganic and organic N, while ECM occurred throughout the range of N and P fraction distribution. I hypothesize that this fungal distribution relates to the capacity of each fungal type to utilize various soil substrates as nutrient sources.

As a secondary study, I investigated the impact that different mycorrhizal types have on root system architecture. Using PCR and sequencing, I identified the different mycorrhiza occurring on roots of each of the three host species. I then used scanned images of each root fragment to determine several topological parameters including altitude, magnitude, and total exterior path-length. Comparisons of the topology of roots colonized by different mycorrhizae indicate that different ECM groups do not have a noticeable effect on the parameters measured. Other comparisons could not be made due to a lack of AM and ERM diversity on the roots tested.

INDEX WORDS: mycorrhiza, nitrogen, phosphorus, topology, architecture, ectomycorrhiza, arbuscular, ericoid.

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CHAPTER 1

INTRODUCTION

Mycorrhizae are structures formed by a symbiotic association between a fungus and a plant root. The fungus that partners with the plant root acquires nutrients, such as nitrogen and phosphorus, from the soil matrix and transfers them to the plant root system. The fungi typically possess biochemical capabilities that allow them to access nutrient sources the plant cannot (e.g. Allen 1987 1991, Marx 1975, Carpenter and Allen, 1988). The fungus provides these nutrients to the host plant in exchange for carbohydrates produced photosynthetically in the leaves of the plant (Smith and Read, 1997). Mycorrhizae are believed to have evolved along with land plants, perhaps as long ago as 456 million years before present in the case of arbuscular mycorrhizae (AM) (Simon et al. 1993). There are several thousand species of fungi that are capable of forming mycorrhiza. These species may be divided into over seven major types, each with unique properties and functions. Understanding how these different types and species of mycorrhizae function and the resulting effects on community structure is an important issue because the mycorrhizal symbiosis is fundamental to the nutrition, growth, and sometimes survival, of most species of plants.

Objective:

The objective of this study was to determine the vertical distribution in soils of the three major types of mycorrhiza, and to determine if this vertical distribution was correlated to soil N and P distribution.

Chapter 1 investigates the vertical distribution of the three mycorrhizal types in a soil supporting *Liriodendron tulipifera, Tsuga canadensis,* and *Rhododendron maximum* at the Coweeta Hydrologic Laboratory, Otto, North Carolina. The assessment of the mycorrhizal community was conducted using DNA based technology, and chapter 1 also tests several methods of DNA extraction from soils to optimize the fungi recovered.

Chapter 2 extends the work accomplished in chapter 1 by determining the nitrogen and phosphorus fraction distribution in the soil studied. Principal Component Analysis is then utilized to test whether mycorrhizal distribution and N and P fraction distribution are correlated.

Chapter 3 analyses the topological effects of mycorrhizal infection. Root fragments from the Coweeta soil were tested for the presence of mycorrhizae by DNA sampling. They were also topologically analyzed, and the topology and type of mycorrhizae colonizing the root was tested to determine if mycorrhizal type had a significant effect on topology.

Literature Review:

The three most widespread types of mycorrhiza are arbuscular, ecto- and ericoid mycorrhiza. Arbuscular mycorrhizae (AM) are the most common mycorrhizal symbiosis (Smith and Read, 1997), and are formed principally by Zygomycete fungi of the order Glomales. AM fungi are endophytes, forming structures within the host root cells. These structures include arbuscles, which are likely to be the site of nutrient and carbohydrate exchange between the plant and fungus (Smith and Read, 1997). Some AM also form vesicles, thought to be storage structures, and coils, which may have an exchange function (Smith and Read, 1997).

Ectomycorrhizae (ECM) are principally basidiomycete or ascomycete fungi, within a variety of orders (Smith and Read, 1997). Ectomycorrhizae do not form intercellular structures, instead they form a mantle, and Hartig net (Smith and Read, 1997). The mantle is a sheath of

fungal tissue, which encloses the root tip, while the Hartig net is fungal tissue that grows between cortical cells. Ectomycorrhizae are also characterized by extensive hyphal growth out into the soil (Agerer, 1995).

Ericoid mycorrhizae (ERM) are ascomycetes in the order Ericales (Read, 1996). ERM fungi produce dense intracellular hyphae, similar to ectomycorrhizae, but lack any mantle (Smith and Read, 1997). ERM roots are characterized by an absence of root hairs and the extensive development of "hair roots" which are anatomically simplified roots typically less than 100μm in diameter (Read, 1996).

There are functional and ecological differences between the fungi that develop arbuscular, ericoid, and ectomycorrhizas that are pertinent to this study. These differences include 1) the extent to which each type produces extra-matrical hyphae; 2) the horizontal and vertical spatial distribution of the mycorrhizal fungi in soils; 3) the ability of the different fungi to utilize various organic and inorganic substrates as nutrient sources; and 4) their impacts on plant root architecture.

Hyphal production and distribution:

The ability of AM, ECM, and ERM to produce extra-matrical hyphae has a direct impact upon their distribution within soils.

Arbuscular mycorrhizal fungi lack the psuedoparaenchymetous structures that are characteristic of ectomycorrhizal rhizomorphs. Extra-radical hyphal development of AM's can be quite extensive, however. Hyphal growth out 20-30μm from an infected root (Warner and Mosse, 1983; Schüepp et.al., 1987) with maximums up to 90μm have been observed (Smith and Read, 1997), corresponding to 7.1 to 250 m m^{-1} colonized root length. Hyphal diameter can vary widely, from 2 to 27μm (Smith and Read, 1997), while hyphal growth rates average anywhere

from 0.2 to 3.0μm per day (Camel et.al. 1991; Scheltema, 1987). Because of the small diameter of extra-radical hyphae they are clearly well adapted to exploring soil pores of a size smaller than those that plant roots may utilize, and hyphae also proliferate around areas of concentrated soil organic matter (St. John et.al., 1983). Soil nutrient levels also affect the extent of hyphae production. There is a negative correlation between soil phosphorus and extra-radical hyphal length of AM fungi (Miller and Jastrow 1992b). The hyphae of AM fungi can form a variety of architectures from very diffuse to densely aggregated (Jakobsen et al. 1992). AM species also can differ in the rate at which they can colonize soil and root.

ECM have the capacity to produce rhizomorphs, which are bundled linear strands of individual hyphae, forming cord-like structures (Agger, 1995). Rhizomorphs can expand up to tens of meters across the forest floor, with absorbing hyphae emanating from them. Rhizomorphs have the capacity to transport nitrogen and phosphorus considerable distances and may act as channels of carbohydrate exchange between plants (Read 1993). Extra-radical mycelia from a variety of ECM forming hyphae may grow 2 to 4 mm d^{-1} on average (Coutts and Nicholl, 1990a; Finlay and Read, 1986a,b,c). Using hyphal length per soil weight as a measurement unit, ECM hyphae range from 2.8 to 6.42 m $gram^{-1}$ of soil and were estimated to increase the nutrient absorbing surface in these soils up to 40-fold (Rousseau et al. 1994). The growth of the mycelium is seasonal, with growth rates at a maximum in late summer, ceasing or greatly reduced during winter months (Coutts and Nicholl, 1990). While these growth rates are on par with growths rates of many plant roots, the density of hyphae better facilitates soil exploration. Estimates of ECM hyphal density are wide ranging from 250 to 8000m per meter of root, depending on the fungal and plant species as well as the soil conditions (Jones et.al, 1991; Read and Boyd, 1986).

The quantification of ERM hyphal development into soils has received little attention. As a result, measurements aren't available in the literature with which to compare the extent of extra-matrical hyphal development of ERM with that of ECM and AM fungi. It is generally thought that ERM hyphal development into the soil is limited, with the majority of ERM hyphae clustered around the "hair-root" structures.

The spatial distribution of mycorrhizal hyphae vertically and horizontally within soils is difficult to measure since the vegetative structures are underground and difficult to view.

It is generally thought that AM fungi are more uniformly distributed in comparison to ECM (Smith and Read 1997). However, other studies contradict this theory. Several studies have demonstrated an aggregate distribution for AM fungi (Klironomos et al., 1993, Friese and Koske 1991), which may be influenced by edaphic factors such as soil moisture (Anderson et al., 1986) or pH (Coughlan et al., 2000). Husband (2002) suggests that there is more host specificity and site preference among AM fungi than previously thought, and that it is likely that AM communities change over time with site conditions. AM fungal community studies have typically been carried out using spore counts to represent community structure. This procedure may be a factor leading to confusion in AM community analysis as many factors effect spore production and diversity (Eom et al., 2000, Morton et al., 1995).

Goodman and Trofymow (1998) sampled fungal root tips from seven unique habitat areas in a forest stand to determine if different ectomycorrhizal types preferred specific substrates. They found that ectomycorrhizae were distributed in a spatially clumped pattern due to the substrate preferences of different ectomycorrhizal species; some ECM preferred mineral soils while others were recovered solely from organic substrates.

Recently, two studies utilized molecular approaches to define the ectomycorrhizal hyphal distribution within soil horizons. Dickie et al., (2002) sampled soil from four layers in a red pine

stand and used Terminal Restriction Fragment Length Polymorphism (T-RFLP) to compare soil derived DNA fragments to sporocarp or root tip DNA. Thirty-six T-RFLP patterns were identified, indicating 36 different species of fungi. Their analyses indicated that soil horizon was a significant factor in ECM distribution, with the most ECM fungi found in the lower litter and the fewest in the B-horizon. They found the most ectomycorrhizae in the litter layer, steadily declining with depth, with predominately *Lactarius*, *Tylopilus* and others found in the litter layer while *Suillus* and *Russula* were found only in the lower soil horizons. Dickie et al. (2002) hypothesized that this unequal distribution arises due to niche differentiation among the mycorrhizal species present, limiting direct competition between different ectomycorrhizal species.

Landeweert et al. (2003) sampled soil from a pine-spruce stand and used cloning and sequencing techniques to determine the species of fungal DNA fragments amplified from the soil samples. Twenty-nine different sequences were identified, 5 of which could be confidently assigned to a mycorrhizal genus. Thirteen of the sequences had high similarity to sequences obtained from root tip samples, and 10 of the soil-derived sequences occurred in the same horizon from which root tip samples were obtained. Landeweert et al (2003) also found that ectomycorrhizas were distributed throughout the soil profile in a clumped pattern. They found *Russula and Suillus* only in the O and E horizon while *Cortinarius, Tylospora, Laccaria* and others were limited to the B horizon.

There has not been significant work done concerning the soil distribution of ERM fungi. We are not aware of any study on the stand or soil based distribution of ERM fungi.

Mycorrhizal substrate utilization:

The ability of mycorrhizae to secrete enzymes into the surrounding soil environment to transform unavailable forms of nutrients into available forms, is one of the more crucial aspects of their ecology. Most fungi and plant root systems produce some degradative enzymes, such as phosphomonoesterases. However, the extent and activity of the enzymes produced by mycorrhizal fungi, as well as the close association of the fungal hyphae with the enzyme substrate, enhances the fungi's ability to acquire significant levels of soil nutrients through this mechanism.

Mycorrhizal production of several different classes of enzyme have been investigated. These include the glycosidases (EC 3.2.1.-), cellulase (EC 3.2.1.4), B-xylosidase (EC 3.2.1.37), and β-glucosidase (EC 3.2.1.21), the phosphoric monoester hydrolases (EC 3.1.3.-) which include alkaline and acid phosphatases (EC 3.1.3.1 and 3.1.3.2), the phosphoric diester hydrolases (EC 3.1.4.-), peptidases (EC 3.4.-.-), and polyphenoloxidase (EC 1.14.18.1).

Glycosidases have been associated with ECM and ERM, though at low levels compared to saprophytic fungi (Colpaert and Van Laere, 1996). Cellulase (endo activity EC 3.2.1.4) and cellulose 1,4 β-cellobiosidase (exo activity EC 3.2.1.91) which converts cellulose to cellobiose have demonstrated activity in mycorrhizal fungal extracts. Cairney and Burke (1998) measured cellulose 1,4 β-cellobiosidase in both ecto and ericoid mycorrhizae, with high activity in the ericoid species (*Hymenoscyphus ericae*) and variable activity in the ectomycorrhizal fungi (*Suillius variegatus, Cortinarius sp., Pisolithus tinctorius*). In this same study β-glucosidase activity, which converts cellobiose in glucose, was also found in both ERM and ECM fungi, with similarly high levels in the ERM fungi and variability in the ECM fungi. Burke and Cairney (1997) purified β-1-4-endoxylanase (EC 3.2.1.8) in *H. ericae* which functions in the decomposition of the hemicellulose Xylan.

Phosphoric monoester hydrolases have been purified from ERM (Pearson and Read 1975; Mitchell and Read 1981), ECM (Alexander and Hardy, 1982; Dighton, 1991), and AM fungi (Joner and Johansen, 2000). Phosphoric monoester hydrolases (or phosphomonoesterases) decompose the ester (C~O~P) bonds found in compounds such as phytate and inositol phosphates. Joner and Johansen (2000) measured phosphomonoesterase activity in the AM fungi *Glomus intraadices* and *G. claroideum.* This AM activity is at the high end of the range demonstrated for ERM and ECM fungi studied by Colpaert and Van Laere (1996). Phosphoric diester hydrolases decompose the diester (C~P~C) bonds of compounds such as DNA and phospholipids. ERM fungi are known to possess Phosphodiesterase activity (Leake and Miles 1996), but has not been measured in other types or species.

ERM fungi (Leake and Read, 1990; Colpaert and Van Laere, 1996; Bending and Read, 1996) and ECM fungi (Colpaert and Van Laere, 1996; Bending and Read, 1996; Tibbett et al, 1999) possess peptidase activity. Peptidases (also called proteases) include exopeptidases and endoprotienases (or protienases). Peptidases cleave peptide bonds or remove amino acids from the terminus of the protien. Peptidase activity has not been demonstrated in AM fungi, and it is unlikely that AM fungi retain the ability to produce peptidases to any important extent.

Polyphenol oxidase activity has been demonstrated in ecto and ericoid mycorrhizae. Polyphenoloxidase is an important enzyme in forest areas that are characterized by plant materials high in phenolics, such as tannins. These substances, found in pine and oak litter among others, releases tannin upon decomposition and forms protein-tannin complexes which are very resistant to any further breakdown. Thus in these forest areas, large percentages of available N are sequestered in this tannin-protein complex. Polyphenoloxidase decomposes the polyphenols allowing peptidases access to the previously protected proteins. Colpaert and Van Laere (1996) assessed the ability of two ectomycorrhizal and one saprophytic fungi to produce

polyphenoloxidase. The saprophytic fungus *Lepista nuda* produced a high level of activity (1.21 nkat/g/d.wt) while the ectomycorrhizal fungus *Suillus bovinus* produced only 0.36 nkat/g/d.wt. The second ectomycorrhizal fungal species *Thelephora terrestris* didn't produce a significant polyphenoloxidase activity. This demonstrates the possibility of a wide range of ability of ectomycorrhizae to produce this enzyme. The ECM fungus *Lactarius controversus*, produces more than twice the activity of polyphenoloxidase than the ERM species *H. ericae* (Bending and Read, 1996).

These studies demonstrate that mycorrhizal fungi have a variable but extensive capacity to produce degradative enzymes allowing the fungi access to the major pools of organic N and P. Wider ranging studies with more species of each mycorrhizal type are needed to determine the capacity of each type of symbiotic fungi to produce these enzymes.

Root Architecture:

Root architecture is comprised of three components: topology, size and position. Topology is defined as the configuration of the components of a system with respect to each other. For plant root systems these components are usually considered to be the segments of roots between branch points. The segments are called links and the branch points are called nodes. The topology or configuration of these components is defined using a variety of measurements. These measurements include:

1. link length - the length of a root between two nodes.
2. lateral branch angle -the angle between the growth vector of the parent and child root.
3. radial branch angle – the angle between the growth vectors of two child roots.
4. link magnitude – each link has a magnitude which is the number of links that has developed from the link of interest.

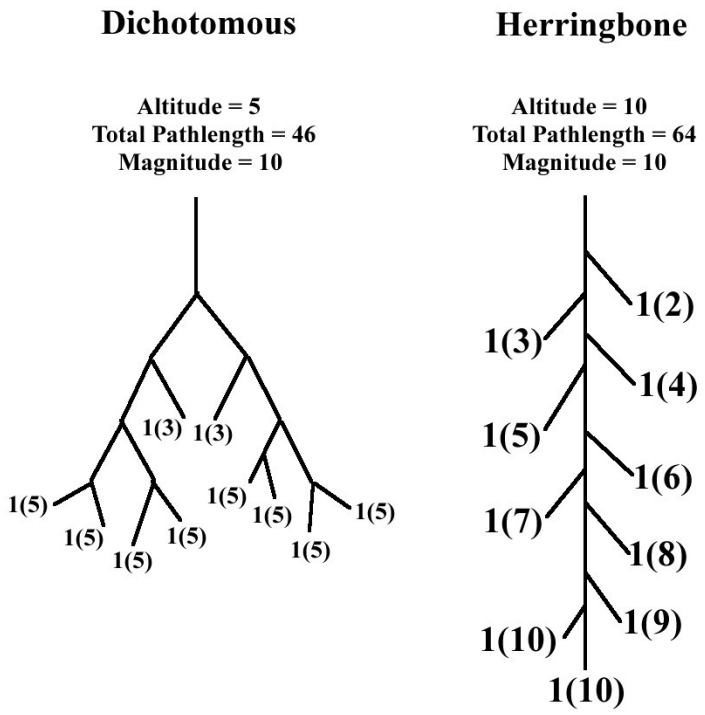
5. link pathlength – this is the reciprocal of link magnitude, pathlength is the number of lengths from the length of interest to the resource sink defined for the root systems under study.
6. altitude – the number of links in the longest individual pathlength from resource sink to the most outer link.

Size is the diameter of each link, and position is where in the soil medium the link is located. Three numbers, the total exterior pathlength, the altitude, and the magnitude can characterize a specific, unique topology (see Figure 1.1). Total exterior pathlength (Pe) is the sum of pathlengths from all sources.

Theoretically, there are two extremes to root topology (Fitter 1985), the "herringbone" topology and the "dichotomous" topology (Figure 1.1). The herringbone type consists of a main tap-root with unbranched lateral roots. The dichotomous system is a highly branched rooting structure with many lateral roots of multiple orders (Fitter 1985). The herringbone type is less transport efficient than the dichotomous due to greater pathlength (Fitter 1987). The herringbone's main axis is required to transport absorbed materials from all other links. The dichotomous system, with is highly branched structure, is more transport efficient in this way, as each link passes its materials through a minimum number of links to reach the sink. Therefore, the dichotomous structure has more exterior links (absorbing links) at a lower total root magnitude; this means the dichotomous system has more absorbing capacity, and transport capacity per unit of root biomass.

Herringbone type topologies are more costly to construct due to a greater number of high magnitude links (Fitter and Stickland 1991). Since xylem conductivity requires an increase in xylem cross sectional area with magnitude, and xylem area is directly proportional to root diameter (Fitter 1987), the herringbone root will have greater diameter links for a given

Figure 1.1 - Examples of the "herringbone" and "dichotomous" topologies. Each root has a magnitude of 10. For the same magnitude, the herringbone topology has greater total exterior path-length and altitude than the dichotomous topology. Numbers not in parentheses represent a single root tip. Number within parentheses represent the pathlength from the point of origin to the root tip.



magnitude than will the dichotomous topology. Therefore, the herringbone system will require more construction and maintenance input at a given magnitude than will the dichotomous. Nielsen, Lynch et al. (1994) measured the carbon cost of the different topologies as respiration (% of total C respired over time). As predicted, the herringbone system was far more costly than the dichotomous system. Larger diameter roots have greater longevity than smaller diameter

roots (Wells and Eissenstat, 2002; Eissenstat et al., 2000). Since the theoretical herringbone topology has greater diameter roots than the dichotomous system at a specific magnitude, the herringbone system will also have greater longevity at a specific magnitude (Fitter 1987).

The impact of the structure of ectomycorrhizae (ECM) on root topology is well known (Gerdemann, 1971). ECM cause root elongation and root hair formation to be suppressed due to the formation of the ectomycorrhizal mantle. The short lateral roots typical of ECM hosts undergo dichotomous branching, forming several orders of laterals and corolloid structures. This increase in branching seems to result from hormonal interaction between the fungus and root (Kaska et al., 1999). Thus, the expected result of ECM colonization is an increase in root branching and a more dichotomous topology better adapted at absorption of materials from the fungal hyphae. However, few studies have utilized topological analysis to investigate the architectural characteristics of ECM roots.

Arbuscular mycorrhizal (AM) fungi seem to modify plant root topology in various ways. Farley and Fitter (1999) found no change in root topology in herbaceous perennials after AM colonization, while Hodge et al. (2000) reported roots of *Plantago lanceolata* increasing production in organic patches upon addition of AM innoculum. Cui and Caldwell (1996) reported decreased growth of *Agropyron desertorum* roots upon addition of arbuscular mycorrhizae, while Torrisi et al. (1999) describes an increase in root density of cotton roots following mycorrhizal colonization. There are conflicting topologies recorded for tree species as well. Pregitzer et al. (2002) found that AM *Liriodendron tulipifera* roots were thick and unbranched with a low specific root length, while AM *Acer saccharum* roots were much thinner and extensively branched. Clearly, there is no uniform topological response of AM roots to mycorrhizal infection. Moreover, Hetrick et al. (1988) suggested that this decreased branching in *Andropogon gerardii*, which occurred in only low phosphorus conditions, might result from

the root system adapting its architecture for soil exploration, relying on the mycorrhizal fungus for absorptive tissue. Hetrick et al. (1988) also suggested that under appropriate soil nutrient conditions, the mycorrhizal fungi might directly control plant root branching by hormone production.

The impact of ericoid mycorrhizae (ERM) on root topology is unknown. Ericoid mycorrhizal roots are referred to as "hair roots" due to their extremely small diameter (Read, 1996). Hair roots form a dense layer of fibrous roots near the soil surface (Dodd et al., 1984), but the impact of the production of these hair roots on topological parameters is unknown.

Conclusion:

A majority of the studies aimed at determining the community structure of mycorrhizae have been hampered by inefficient technology, utilizing fruiting structures or spores in an attempt to determine fungal distribution. This study utilized DNA based protocols to determine the vertical distribution in soil of the three major types of mycorrhizae (AM, ECM, ERM). We also determined N and P fraction distribution in the soil and tested whether this fraction distribution was correlated with the mycorrhizal distribution. Additionally, we studied the impact mycorrhizal colonization has on root system architecture for three tree species.

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CHAPTER 2

THE VERTICAL DISTRIBUTION OF ERICOID, ECTO-, AND ARBUSCULAR MYCORRHIZAE IN A MIXED SPECIES FOREST SUSTAINING ALL THREE TYPES[1]

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ABSTRACT

The spatial distribution of mycorrhizal fungi in soils plays a key role in their ability to provide their symbiotic hosts with nutrients. However, details about this spatial distribution, especially for arbuscular and ericoid mycorrhizae. are few. We investigated the vertical distribution of ericoid (ERM), ecto- (ECM), and arbuscular (AM) mycorrhizal fungi in a soil of the Coweeta Hydrologic Lab, Otto, N.C.. The soil contained host plant roots of all three mycorrhizal fungi, *Liriodendron tulipifera* (AM). *Tsuga canadensis* (ECM), *Rhododendron maximum* (ERM). We utilized PCR, cloning and DNA sequencing to determine mycorrhizal fungal distribution. Soil was collected from three soil horizons (O,A,B) from 16 plots. In order to produce an accurate picture of the mycorrhizal community in this soil, five different DNA extraction procedures were tested for their ability to provide quality and quantity of DNA. A commercially available kit, Fast DNA Spin Kit from QbioGene, performed the best for the soil studied. DNA extracts produced from this kit were utilized in cloning and sequencing procedures. Sequences were identified using BLAST searches and Maximum-Likelihood analysis. The statistical significance of the mycorrhizal spatial distribution was tested using Fisher's Exact Test. Our results indicate that the three mycorrhizal types are differentially distributed throughout the soil horizons. ERM fungi occurred predominately within the O soil horizons and AM fungi occurred mainly in B soil horizons. The majority of ECM fungi were located within the A soil horizon and ECM fungi were found in the O and B a well. We hypothesize that this fungal distribution relates to the capacity of each fungal type to utilize various soil substrates as nutrient sources.

Introduction

The distribution and growth of forest trees is controlled by a large number of factors, such as light, water and seed dispersal patterns (Ward and Parker, 1989). However, other, less well-understood factors also likely play a role, such as mycorrhizal fungi. This symbiosis between roots and fungi benefits trees by increasing their capacity to acquire soil nutrients. It is also clear that different mycorrhizal fungi, such as ericoid (ER), ecto- (ECM), and arbuscular (AM) have different capacities for improving a tree's rate of nutrient acquisition (Read and Perez-Moreno, 2003). This variation, which likely plays a role in the productivity of forest trees (Francis and Read, 1994), may be influenced by several factors. These may include the biochemistry of the symbiotic interaction, or physical aspects such as the distribution of the fungal mycelium throughout the soil.

Several studies have described the distribution of mycorrhizal fungi throughout different substrates and soil depths. Goodman and Trofymow (1998) sampled mycorrhizal root tips from 7 unique forest habitat areas to determine if different ECM types preferred specific substrates. They found that ectomycorrhizae were distributed in a spatially clumped pattern; some ECM fungi preferred mineral soils while others were recovered solely from organic substrates. Landeweert et al., (2003) also found that ECM fungi were distributed throughout the soil profile in a clumped pattern. *Russula spp.* and *Suillus spp.* were observed only in the O and E horizon while *Cortinarius spp., Tylospora spp., Laccaria spp.* and others were limited to the B horizon. Dickie et al. (2002) found the most ECM fungi in the litter layer, the number of species steadily decreased with increasing depth. *Lactarius spp.,* and *Tylopilus spp.* were predominant in the litter layer, while *Suillus spp.* and *Russula spp.* were found only in the lower mineral soil horizons. Dickie et al. (2002) hypothesized that niche differentiation gives rise to this unequal

distribution among ECM fungi, limiting direct competition between different ectomycorrhizal species.

The most prevalent type of mycorrhizae are AM; two-thirds of plants are capable of forming mycorrhizae of this type (Fitter and Moyerson 1996). Several studies have demonstrated a spatially aggregated distribution for AM fungi (Klironomos etal 1993, Friese and Koske 1991), which may be a result of edaphic factors such as soil moisture (Anderson et al 1986) or pH (Coughlan et al. 2000). However, we are not aware of any study of AM hyphal distribution among different soil depths.

Even less information is available about the community composition and dynamics of ERM fungi. ERM form on plants of the Ericaceae, and many ERM fungi possess the ability to degrade recalcitrant organic substrates (Cairney et al. 2000). The number of ERM species is unknown (Perotto et al. 2002) and the taxonomy of the known species is debatable (Vralstad et al., 2000; Sharples et al. 2000a) making ERM fungal community studies even more problematic. We are aware of no study concerning the stand-based or soil-based distribution and dynamics of ericoid mycorrhizae.

Recently, two studies have been published utilizing molecular approaches to define the ECM fungal hyphae distribution within soil horizons. Dickie et al. (2002) sampled soil from four layers in a red pine stand and used T-RFLP to compare soil derived DNA fragments to sporocarp or root tip DNA. Their analysis indicated that soil horizon was a significant factor in ECM distribution; most ECM fungi were found in the lower litter layer , and the fewest in the B-horizon. Landeweert et al. (2003) sampled soil from a pine-spruce stand and used cloning and sequencing techniques to determine the species of fungal DNA fragments amplified from the soil samples. Twenty-nine different sequences were identified, 5 of which could be confidently assigned to a ECM fungal genus. Thirteen of the sequences had high similarity to sequences

obtained from root tip samples, and 10 of the soil-derived sequences occurred in the same horizon from which root tip samples were obtained. The distribution of the different sequences within the soil profile was non-random, indicating that functional differences among species of ECM fungi may be impacting their distribution. While neither study sought to determine the causation of the non-random distribution of ECM fungi among soil horizons, the results of both suggest that there are likely functional differences between ECM species that influence their vertical distribution. These functional differences might include fungal ability to utilize various forms of organic nitrogen and phosphorus (Read and Perez-Moreno, 2003) as nutrient sources, or sensitivity to various environmental factors such as pH or moisture content (Kernaghan and Harper, 2001). More studies, such as these, are necessary to determine how other ECM fungi, as well as AM and ERM fungi, are distributed in a broad array of soils and under a variety of conditions to better understand mycorrhizal fungal community dynamics.

Until recently, methodological challenges limited the level of resolution attainable regarding the community structure of mycorrhizal fungi, especially the soil hyphal component of the fungi. With new technologies, as used by Landeweert et al. (2003) and Dickie et al. (2002), we are improving our understanding of the hyphal distribution of mycorrhizal fungi. However, there are still pitfalls in the molecular approach to mycorrhizal analysis. DNA extraction, amplification, and cloning all have the capacity to bias the perceived diversity of fungal DNA in a sample, through preferential extraction or amplification (Krsek and Wellington, 1999). There are a wide range of protocols available for extracting DNA from soils; each protocol has benefits and drawbacks (Martin-Laurent et al. 2001). The first objective of this study was to test different extraction methodologies, and to determine which protocol performed best with our soil, in order to maximize the quantity and quality of DNA extracted. A second objective was to develop a technique to utilize molecular protocols to evaluate the hyphal distributions of each of the three

major mycorrhizal types, and determine if the mycorrhizal types and the different species of each were distribution non-randomly between soil horizons.

We hypothesized that the distribution of the different mycorrhizal types is related to the ability of each type to acquire nutrients from different substrates. We also hypothesized that ERM fungi would occur predominately in the litter layers (O horizon) of the soil, while ECM fungi would primarily occur in the A soil horizon, and AM would occur in the B soil horizon.

We extracted DNA from 3 soil horizons in a forested stand comprised by all three major types of mycorrhizae (ECM, AM, and ERM). Using DNA extracted from three soil horizons, we attempted to determine the distribution of each type of mycorrhizae, in order to evaluate whether different mycorrhizal types preferred different soil horizons.

METHODS

Site Characteristics:

The study sites are located at the Coweeta Hydrologic Laboratory located in Otto, North Carolina (35°02'44"N, 83°27'09"W). Average monthly precipitation at Coweeta Hydrologic Laboratory is 151.8mm. Highest rainfall typically occurs between December and March. The coldest months are December through February with an average January temperature of 3.3°C. The warmest months are June through August, with an average July temperature of 21.6°C. Mean annual temperature is 12.6°C. The soils at our study sites fall under the Cullasja-Tuckasegee complex of soil series with the Chandler and Saunook series as competitors. The Tuckasegee soil is a fine-loamy, isotic, mesic Humic Dysrudept. It consists of well drained soils on gently sloping to very steep benches, foot slopes, toe slopes, drainageways and fans in coves in the Southern Appalachian Mountains. These soils formed in colluvium derived from materials weathered from igneous and metamorphic crystalline rocks such as granite, mica gneiss,

hornblende gneiss, and schist. Our study sites were located on southeast facing 10-40% slopes including toe, foot, and shoulder slope positions. In general, all plot locations were well drained with the O_i ranging from 10 to 40 cm and composed of primarily rhododendron and oak leaves. The O_e ranged from 1-3 cm and was very dark brown (7.5YR2.5/2) to dark reddish brown (5YR3/2). O_a layers were generally humic in nature with many fine roots. The boundary between the O_a and A soil horizons varied between clear and gradual, in some instances it was impossible to determine a clear O_a/A boundary. The A horizons were loams to fine sandy loams and varied from very dark brown (7.5YR2.5/2) to dark yellowish brown (10YR4/4) and were between 20-30 cm in depth with weak fine granular structures. The A horizons contained many large roots and generally had clear wavy boundaries. The B horizons were silty loams to silty clay loams, ranging in color from yellowish brown (10YR5/6) to brown (7.5YR4/6), and were between 20-40 cm in depth. The B horizons generally had a weak medium subangular blocky structure with 10% cobbles.

The sampling plots were located in a cove forest area with a *Liriodendron tulipifera* overstory, *Tsuga canadensis* in the sub-dominant canopy to understory position, and *Rhododendron maximum* understory. *L.tulipifera* is associated with arbuscular mycorrhizae (AM), *T. canadensis* forms ectomycorrhizae (ECM), and *R. maximum* forms ericoid mycorrhizae (ERM). Four sampling plots of $25m^2$ each were located within the cove forest areas. Within each plot, four subplots were randomly located. Within each subplot, soil samples were collected from three horizons. The sampling involved digging a soil pit, $900cm^2$ in area, through the B-horizon and collecting the O, A, and B horizons. In cases where the O/A boundary was difficult to distinguish, horizons were separated where mineral soil predominated. Soil samples were stored in plastic bags and frozen with dry ice for the trip back to the laboratory.

Sample preparation:

In preparation for DNA extraction, soil samples were cleaned of root fragments by hand, air dried for 3 days, ground with a mortar and pestle, passed through a 150μm sieve, then freeze-dried and stored at -20°C. O horizons samples were also ground with a Wiley mill before sieving. Roots removed from the soil samples were freeze-dried and stored for subsequent DNA extraction and analysis.

DNA Extraction from Soils:

Five protocols were tested for efficiency in DNA extraction and optimum PCR amplification.

Protocol 1 utilized a hot SDS lysis in a phosphate buffer followed by overnight polyethylene glycol extraction (Selenska and Klingmuller, 1991). Further purification was performed using ethanol precipitation and the use of Promega's Wizard clean-up kit because of excessive humic contamination.

Protocol 2 utilized Polyvinylpolypyrrolidone (PVPP)/Polyethylene glycol (PEG)4000/Glucanex pretreatment with SDS/proteinase K lysis and potassium acetate and glassmilk purification (Porteous and Armstrong, 1991). PVPP is used to absorb unwanted phenolics, PEG4000 precipitates with tannins and other compounds, and glucanex (Novozymes, Inc.) is an enzyme that degrades fungal cell walls. SDS dissolves cell membranes, proteinase K degrades protein contaminants; potassium acetate is used to precipitate SDS. The CsCl density gradient centrifugation step was skipped and extracts purified directly with Glassmilk, as suggested by Porteous and Armstrong (1991).

Protocol 3 involved the use of QbioGene's FAST DNA from Soil SPIN Kit (QbioGene Inc. 2003). The kit incorporates bead beating in detergent, followed by guanidine isothiocyanate purification, which breaks protein-DNA bonds, and ethanol washing.

Protocol 4 utilized a skimmed milk pretreatment with SDS lysis and phenol chloroform purification followed by ethanol precipitation (Garcia-Pedrajas and Bainbridge, 1999). The skimmed milk binds contaminants such as polyphenols, the phenol denatures proteins and the final extraction with chloroform removes traces of phenol. To remove salts, the pellet is washed with 0.5-1.0 ml of 70% ethanol, spun again, the supernatant decanted, and the pellet dried. Garcia-Pedrajas and Bainbridge (1999) "simplified procedure" was followed using the 4.8% skimmed milk solution. With samples of a higher organic matter content the volume of skimmed milk solution failed to adequately wet the sample. In these instances a greater volume of skimmed milk solution, up to 500µl was added until centrifugation provided a useable supernatant volume.

Protocol 5 involved the use of hot SDS and CTAB lysis in a phosphate buffer followed by bead beating with phenol/chloroform/isoamyl purification, isopropanol precipitation and Wizard minicolumn purification (Baek and Kenerley, 1998). Cetyltrimethylammonium bromide (CTAB), is a cationic detergent, that complexes with proteins and polysaccharides and helps precipitate these from extracts. Phenol/chloroform purification is normally used to remove proteins from extracts.

DNA Quantification:

Amount of DNA in each extract was determined two ways, spectrophotometrically and fluorescently. Absorbance at 230, 260, and 280nm was measured to determine DNA quantity as well as amount of contamination by proteins and carbohydrates, using a Beckman-Coulter DU640 spectrophotometer. Fluorimetry was accomplished using PicoGreen DNA marker from Molecular Probes. Fluorescence was measured on a BioTek FLx320 microplate fluorimeter following the manufacturer recommendations.

PCR Efficiency:

To determine if the extraction procedures provided a sample pure enough to effectively amplify with multiple primer sets, each sample was PCR amplified using universal fungal primer pair ITS1F/ITS4 (Gardes and Bruns, 1993). The PCR was carried out in a thermocycler (MJ Research PTC-200, Waltham, Mass.), using 2.5U Fisherbrand Taq Polymerase, 1.5ul 25mM $MgCl_2$, and 5µl of extraction template diluted 1000x in a 25µl reaction volume using the following thermal cycling protocol: 94C, 35s.; 53C, 55s.; 72C, 30s + 5s/cycle; repeated for 34 cycles.

The products were run on an electrophoresis gel stained with Gelstar from Molecular Probes. The brightest bands were assumed to have the least inhibition from contaminants. Those extracts lacking amplification of the fragments of the appropriate size were considered too contaminated to utilize further.

PCR:

Once an extraction procedure was chosen, six different primer combinations were utilized to amplify the range of ECM, ERM, and AM fungi. A primer set that has been utilized extensively for amplification of ECM and ERM, ITS1F/ITS4 (Chen and Cairney 2002; Gardes and Bruns, 1993) was used to amplify the ECM and ERM taxa. GLOM5.8R, and GIGA5.8R in combination with ITS1F and ARCH1311, ACAU1660, and LETC1670 in combination with ITS4, were utilized to amplify the DNA of various AM taxa. Redecker (2000) developed these primer sets to amplify AM fungi from root samples. The GLOM5.8R/GIGA5.8R/ITS1F combination amplifies the *Gigasporaceae* and the *G. mosseae/interadices* group while the ARCH1311, ACAU1660, LETC1670, ITS4 combination amplifies the *Acaulosporaceae, Glomus enticatum/clarodium* group, and the *A. germannii* and *G. occultum* groups (Redecker 2000). The PCR protocol followed that of Redecker (2000).

DNA Extraction from roots:

In order to compare soil fungal DNA sequences with root fungal DNA sequences, as well as to clearly identify plant species, DNA was extracted from the root samples using a protocol developed by Karen (1998), followed by PCR amplification with the primers described above. Several root samples were taken from each soil sample for a total of 109 roots. Several root tips were removed from each root sample, freeze dried and the DNA extracted. A total of 150 root tips were analyzed. Sequences obtained from root samples were aligned with soil derived sequences using the Seqman program from Lasergene (DNAStar Inc., 2003). Sequences with greater than 95% similarity were considered to be matches.

Analysis of PCR Products:

PCR products from soil DNA extracts were cloned using Invitrogen's TOPO-TA 2.1 cloning kit (Invitrogen, 2003) following the manufacturer instructions. Approximately 30 clones per PCR reaction were collected. The clones were then mini-prepped and the DNA sequenced using an ABI 3700 sequencer with ABI BigDye version 2 or 3 chemistry (Applied Biosystems, 2003). PCR products from roots were purified and then sequenced the same as the soil derived products.

Sequence Identification:

Sequences were analyzed using the GCG Wisconsin Package Sequence Analysis software (Wisconsin Package 10.2, Genetics Computer Group, 2001). The GelAssemble module was utilized to compare sequences to each other in order to merge duplicates, remove poor sequences, and to trim vector sequences from the fungal sequences. The sequences were then individually viewed and adjustments were made to make the sequence better match the sequence trace curves. BatchBlastX was then utilized to compare the sequences to the National Center for Biotechnology Information (NCBI) database. Those sequences showing close matches to fungal

species were retained, ClustalX was used to align the sequences to each other and their nearest Blast matches, as well as a variety of known fungal species. Aligned sequences were then entered into the PAUPSearch/PAUPDisplay module of GCG, and a phylogenetic tree was constructed using the maximum-likelihood criterion.

Analysis of mycorrhizal type distribution:

Once each sequence was identified to mycorrhizal type, the number of occurrences within each soil horizon were analyzed utilizing Fisher's Exact Test (Fisher, 1973) with SAS 8.2 (SAS, 2003).

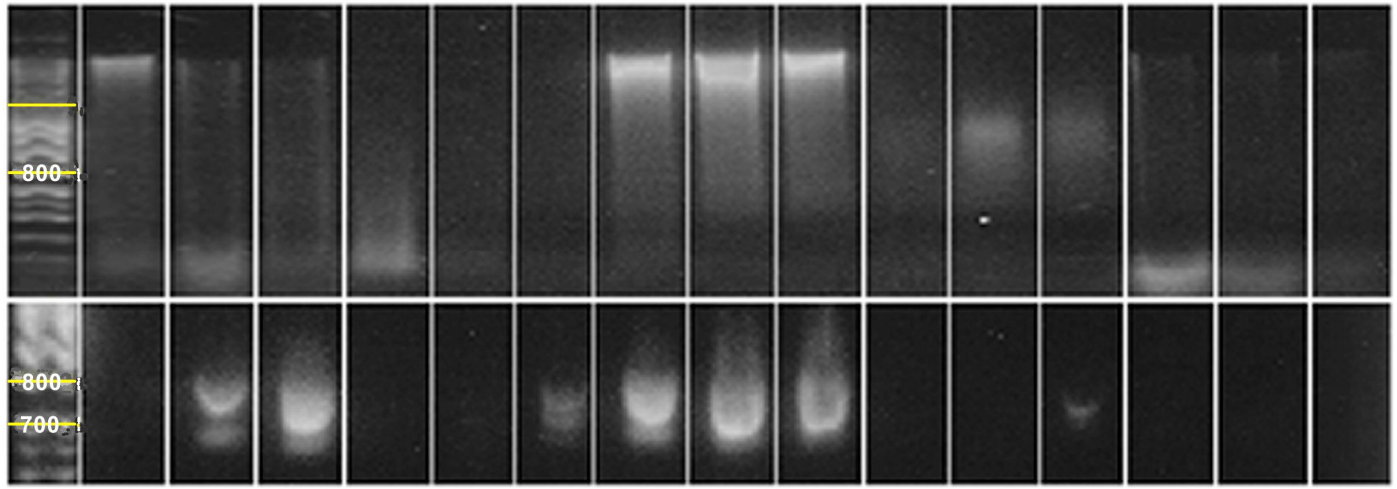
RESULTS

DNA Extraction:

The QbioGene kit (protocol 3) outperformed each of the other extraction methods tested, in all categories measured (Figure 2.1). The QbioGene kit extracted more DNA per gram soil than the other extraction methods for each of the three soil horizons. The skimmed milk protocol was also effective in extracting DNA from each horizon, but PCR amplifications from these extracts were poor, with only the O horizon extract producing any amplification product. The QbioGene protocol produced an extract with the highest 260nm/280nm absorbency ratio indicating that this protocol was effective in removing proteins from solution. On average, the skimmed milk protocol had the highest A260/230 ratio. This ratio indicates that the skimmed milk protocol removed a majority of carbohydrates from solution. On average the QbioGene protocol had the second highest A260/230 ratio. Since PCR amplification is the ultimate goal

Figure 2.1 - Picogreen, spectrophotometric and gel based analysis of 5 different DNA extraction methods (Polyethylene glycol precipitation, protocol 1; method of Porteous and Armstrong (1991), protocol 2; QBioGene soil DNA extraction kit, protocol 3; skimmed milk procedure from Pedrajas et al. 1999, protocol 4; Baek and Kinerley 1998; protocol 5) on O, A, and B horizon soil from a cove hardwood forest in the Coweeta Hydrologic Laboratory, Otto, N.C.. The A260/280 measurement is the ratio of absorbance between 260 and 280nm, the A260/230 measurement is the ratio of absorbance between 260nm and 230nm. The DNA Extractions row displays the images of representative gels from each extract procedure. The PCR Amplification row displays images of representative gels of products resulting from PCR of each extract utilizing the primer pair ITS1F/ITS4.

Protocol #	1			2			3			4			5		
Horizon	B	A	O	B	A	O	B	A	O	B	A	O	B	A	O
Picogreen Assay (ug DNA/g soil)	0.342	0.267	0.274	0.413	0.176	0.461	1.496	2.365	3.101	1.126	1.752	1.147	0.499	0.795	1.486
Spectrophotometric Analyses A260nm/280nm	1.533	1.637	1.530	0.134	0.174	0.113	1.803	1.972	1.890	1.264	1.184	1.442	1.605	1.579	1.486
Spectrophotometric Analyses A260nm/230nm	0.161	0.620	0.208	0.044	0.040	0.071	0.713	0.723	0.727	0.710	0.755	1.232	0.686	0.598	1.160



after extraction, the QbioGene protocol was far superior to the other protocols because the PCR product produced a bright band without significant smearing or mis-amplification. The other protocols produced significantly poorer PCR bands. Based upon the results of the extraction tests, we chose to utilize the QbioGene protocol for subsequent studies.

Sequencing Results:

From the 48 soil samples collected, 1440 clones were processed, producing 128 unique fungal sequences that were recovered and analyzed utilizing Genbank's Blast utility. Of these, 42 of the sequences were possibly mycorrhizal fungi (Table 2.1). The phylogenetic tree created with the sequences (Figure 2.2), their closest Blast match, and several known mycorrhizal fungal sequences revealed that most of the sampled sequences fall clearly within a mycorrhizal group. A few sequences, however, fall outside a known mycorrhizal group, and were not further analyzed. The phylogenetic tree enabled us to limit the 42 sequences to 18 groups or operational taxonomic units (OTU)(Table 2.2).

Soil OTU Distribution:

Fisher's Exact Test (Fisher, 1973) indicates that the distribution of mycorrhizal fungal hyphae within the soil horizons is non-random (p-value < 0.0001). There were 112 recoveries of mycorrhizal DNA from the 48 soil samples. Of these 112 recoveries, 97 were ECM fungi DNA, 6 were AM fungi DNA, and 9 were ERM fungi DNA. The majority of ericoid mycorrhizae (67%) were recovered from the O soil horizon, 75% of ectomycorrhizae were recovered from the A horizon, while 83% of the arbuscular mycorrhizae were recovered from the B horizon (Table 2.3).

Table 2.1 - Nearest BLAST matches for each unique sequence obtained from soil DNA extracts. DNA Extracts were obtained from soil collected from plots located within the Coweeta Hydrologic Laboratory, Otto, N.C. during August 2002. Plots were within cove forest sites populated by *Liriodendron tulipifera, Tsuga canadensis* and *Rhododendron maximum*. DNA extraction involved the use of the FAST-SPIN kit for Soil (QbioGene, Inc. 2002).

Soil Retreived Sequence #	Closest Blast Match	Accession no.	e-value	percent match
1	*Cortinariaceae* mycorrhizal sp.	AF430290	2.00E-87	95
2	*Cortinariaceae* mycorrhizal sp.	AF430290	3.00E-92	99
3	*Cortinariaceae* mycorrhizal sp.	AF430290	2.00E-89	97
4	*Cortinariaceae* mycorrhizal sp.	AF430290	5.00E-74	91
5	*Clavulinaceae sp.*	CSP 534708	-117	99
6	*Clavulinaceae sp.*	CSP 534708	-135	100
7	*Clavulinaceae sp.*	CSP 534708	-142	100
8	*Clavulinaceae sp.*	CSP 534708	-148	100
9	*Tomentella sp.*	TOM534916	-124	100
10	*Tomentella sp.*	TOM534916	-115	99
11	*Russula virescens*	AY061728	-103	98
12	*Russula virescens*	AY061728	-127	99
13	*Russula virescens*	AY061728	-136	97
14	ascomycete leaf litter fungi	AF502802	-121	100
15	unidentified ascomycete	FA8279453	3.00E-79	95
16	*Amanita vaginata*	AB015693	3.00E-85	96
17	*Amanita vaginata*	AB015693	2.00E-87	97
18	*Amanita vaginata*	AB015693	2.00E-92	99
19	*Amanita vaginata*	AB015693	4.00E-97	99
20	*Clavicorona taxophila*	AF033344	2.00E-83	96
21	*Clavicorona taxophila*	AF033344	3.00E-73	95
22	*Endogone pisiformis*	AF006511	-59	99
23	*Endogone pisiformis*	AF006511	-23	95
24	*Trichloma myomyces*	AF287841	-126	100
25	*Panus rudis*	PRU59086	8.00E-61	98
26	*Omphalina velutipes*	OVU66455	2.00E-55	98
27	*Inocybe relicina*	AY038324	5.00E-19	94
28	Homobasidiomycete sp.	HSP534714	2.00E-51	97
29	*Hebeloma* mycorrhizal isolate	AF432845	9.00E-85	95
30	*Serpula lacrymans*	AJ536023	0	100
31	*Russula atropurpurea*	AF418618	0	100
32	*Suillus bovinus*	AJ419215	0	100

Table 2.1
continued

33	*Tricholoma sp.*	AY097046	8.00E-70	99
34	*Pleurotus pulmonaris*	PPU60648	5.00E-74	97
35	*Phaeotellus griseopallidus*	PGU66436	8.00E-70	98
36	*Acaulospora sp.*	ASP541799	8.00E-82	97
37	*Acaulospora sp.*	ASP541799	2.00E-67	95
38	*Tomentella sp.*	TSU83481	0.006	87
39	*Glomus sp.*	AY035654	2.00E-070	97
40	*Hymenoscyphus ericae*	AY046963	-56	96
41	Mycorrhizal ascomycete of *Rhododendron*	AB089667	4.00E-88	99
42	*Hymenoscyphus sp.*	AF252835	5.00E-74	90

Figure 2.2 – Phylogenetic tree created from the DNA sequences extracted from soil samples. 2a) diagram representing the entire phylogenetic tree. 2b,c,d) Exploded views of each phylogenetic tree section as shown in figure 2a. The unrooted phylogenetic tree was created using the maximum parsimony method with the PAUP/GCG software.

Figure 2a

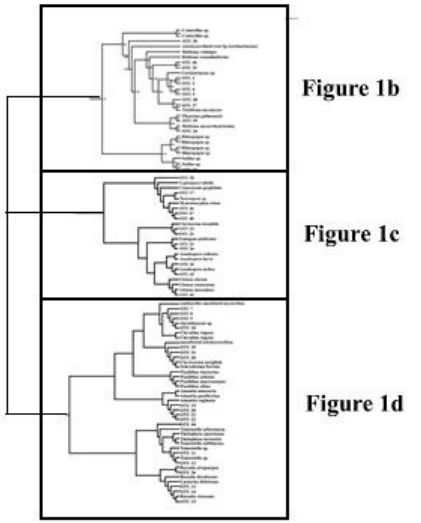
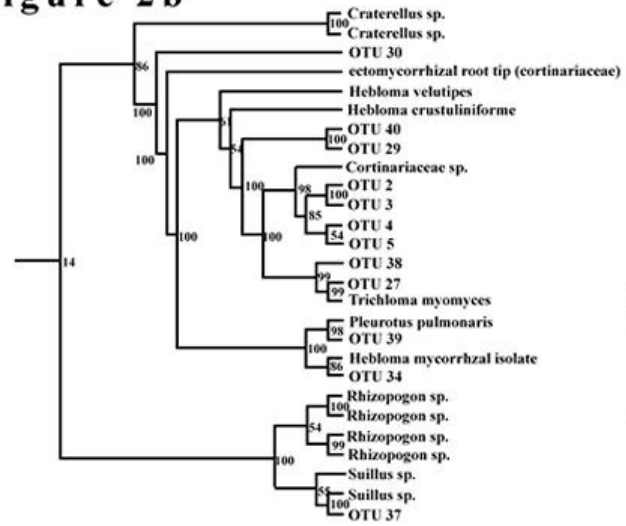


Figure 2b



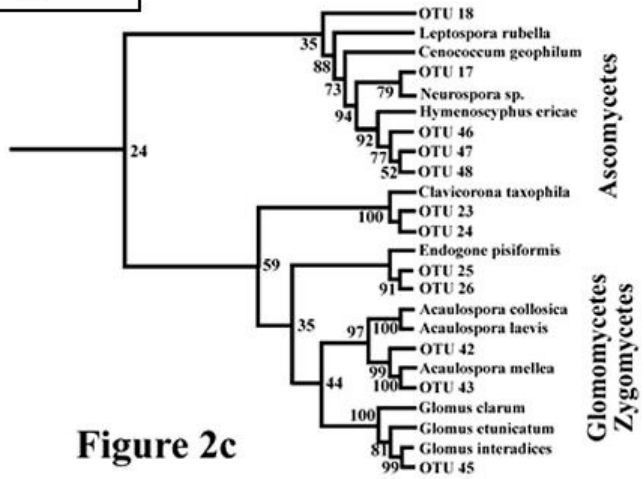


Figure 2c

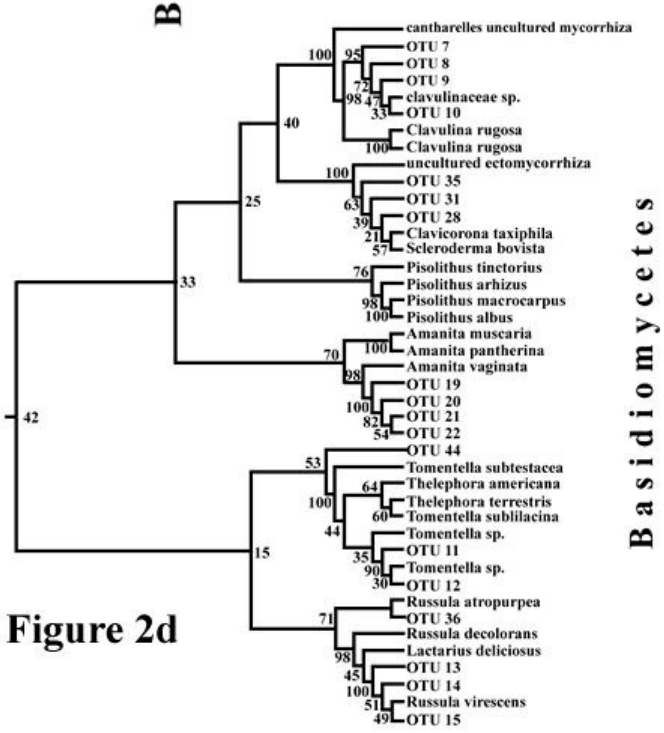


Figure 2d

Table 2.2 - Final OTU identities and mycorrhizal type groupings for the soil derived mycorrhizal DNA. DNA Extracts were obtained from soil collected from plots located within the Coweeta Hydrologic Laboratory, Otto, N.C. during August 2002. Plots were within cove forest sites populated by *Liriodendron tulipifera, Tsuga canadensis* and *Rhododendron maximum*. DNA extraction involved the use of the FAST-SPIN kit for Soil (QbioGene, Inc. 2002).

Soil OTU #	Identity	Mycorrhizal Type
1	*Cortinariaceae* mycorrhizal sp.	ECM
2	*Clavulinaceae sp.*	ECM
3	*Tomentella sp.*	ECM
4	*Russula virescens*	ECM
5	*Amanita vaginata*	ECM
6	*Clavicorona taxophila*	ECM
7	*Endogone pisiformis*	AM
8	*Trichloma myomyces*	ECM
9	*Inocybe relicina*	ECM
10	*Hebeloma* mycorrhizal isolate	ECM
11	*Russula atropurpurea*	ECM
12	*Suillus sp.*	ECM
13	*Tricholoma sp.*	ECM
14	*Acaulospora sp.*	AM
15	*Glomus sp.*	AM
16	*Hymenoscyphus ericae*	ERM
17	Mycorrhizal ascomycete of *Rhododendron*	ERM
18	*Hymenoscyphus sp.*	ERM

Table 2.3 - Number of recoveries of each mycorrhizal type within the three soil horizons (AM-arbuscular mycorrhiza, ECM-ectomycorrhiza, ERM-ericoid mycorrhiza). Numbers in parentheses indicate percentage of each mycorrhizal type found in each soil horizon.

	Soil Horizon			
Mycorrhizal Type	O	A	B	Total No.
AM	0 (0)	1 (16)	5 (83)	6
ECM	8 (8)	73 (75)	16 (16)	97
ERM	6 (67)	3 (33)	0 (0)	9

Fungal Identification from Roots:

We recovered a smaller number of fungi from root systems than from the soil samples (Table 2.4). The amplification success rate from roots was 65%, producing 97 PCR products. Sequencing efficiency was 95%, providing 93 sequences from the 97 amplified, of which 67% matched a soil OTU. In general, each fragment had a single OTU present, and the root tip samples were dominated by several OTU's (Table 2.5).

DISCUSSION

This study is the first attempt of which we are aware to evaluate the distribution of the three main types of mycorrhizal fungi in soil. We chose to utilize PCR/sequencing of the ribosomal ITS region because of its wide use in mycorrhizal research, providing a variety of protocols to choose from and an extensive ITS DNA reference database. We consider this methodology to be the most promising technique for large-scale mycorrhizal sampling. However, several issues need to be addressed before this protocol is usable on a large scale.

Soil Sampling:

First, the sampling method we utilized was time consuming and inefficient. While the acquisition of soil from the face of soil pits ensured the source of each sample, the time required to obtain the material and the disruption to the study site prohibits the use of this technique for larger scale sampling. We initially attempted to use soil cores as our source of material, but we required greater accuracy in determining horizon location than we considered possible with the soil cores. However, we believe that more careful evaluation and processing of soil cores would provide material as accurately assigned to a soil horizon as soil pits would, and be much more efficient and less destructive.

Table 2.4 - Number of recoveries of each mycorrhizal type within the root samples (AM-arbuscular mycorrhiza, ECM-ectomycorrhiza, ERM-ericoid mycorrhiza). Numbers in parentheses indicate percentage of each mycorrhizal type found in each soil horizon.

	Soil Horizon			
Mycorrhizal Type	O	A	B	Total No.
AM	2 (7)	22 (73)	6 (20)	30
ECM	12 (31)	18 (46)	9 (23)	39
ERM	2 (8)	15 (63)	7 (30)	24

Table 2.5 - Sequences recovered from root fragments and their possible identity and mycorrhizal type. The "Matching OTU" column indicates either the identity of the closest BLAST match or the soil derived OTU with which the root sequence aligned using Lasergene's Seqman program. E-values for BLAST matches are given. For sequence matches to root OTU's, the percent matching identity level is >90%.

Root sequence	Matching Soil OTU	BLAST match and ascension #		Mycorrhizal Type
1	OTU 4 (*Russula virescens*)			ECM
2	OTU 5 (*Amanita vaginata*)			ECM
3	OTU 7 (*Endogone pisiformis*)			AM
4	OTU 18 (*Hymenoscyphus sp.*)			ERM
5	OTU 3 (*Tomentella sp.*)			ECM
6	Uncultured mycorrhizal fungus	e^{-92}	AY394903	ECM
7	*Cenococcum geophilum*	$5e^{-81}$	AY112935	ECM
8	*Suillus tomentosus*	$9e^{-88}$	AF323117	ECM

Extraction:

There was tremendous variation in the efficiency of the DNA extraction procedures (Figure 2.1). Each protocol tested in this study had been previously used for DNA extraction from various soils. The fact that there was such large variation in the amount, quality and usability of DNA extracted using each procedure is an indication of the effect that soil variability can have on DNA extraction efficiency. Even within our single soil type there were clear differences in protocol efficiency between horizons. We expected extractions from the A horizons to be most difficult, as humic materials would most likely be more concentrated within this layer (Sjostedt et al., 1997). However, there were no clear trends in extraction efficiency with soil depth (Figure 2.1). It is clear that if we simply settled upon a single extraction protocol, without testing its results against other procedures, we might well have limited the quantity and presumably the diversity of DNA extracted from the soils, limiting our ability to detect mycorrhizal species.

As our data show, the extraction method chosen to recover the DNA material from the soil can influence the quantity and quality of the DNA. Choosing the proper method may greatly influence the success of a study. Since this research was completed several new kits have been released to extract and purify DNA from soils. Given the success we had with the single kit utilized in this paper, it is likely that the continued development of these kits will improve the efficiency of the soil DNA extraction process.

PCR:

The ITS1F/ITS4 primer set amplified a variety of mycorrhizal types, including ERM and AM, while primarily amplifying basidiomycete ECM. The primer set developed to amplify AM (GLOM5.8R, and GIGA5.8R in combination with ITS1F and ARCH1311, ACAU1660, and LETC1670 combined with ITS4) did a poor job, very few Glomomycetes and Zygomycetes were

amplified with this primer set, while several different Basidiomycetes were amplified. This unexpected lack of the primer specificity resulted in a smaller number of AM fungi amplified from the soil samples in comparison to ectomycorrhizae. We don't know why this primer set performed so poorly with our soil samples. This primer pair has not been previously used on soil derived DNA samples, but instead, only with DNA from root extracts, and while it might amplify Glomomycetes and Zygomycetes from roots, the primers may not be specific enough to be used in samples where DNA from a variety of fungal types is present. Redecker (2001) indicates that these primer pairs are sensitive to template concentration, becoming more non-specific with higher DNA concentrations. Given the high diversity of DNA in soil extracts, these primers may be more suitable for the amplification of less diverse soil DNA samples where DNA concentration could be controlled.

Ericoid mycorrhizae were also poorly represented within the PCR products. We were relying upon the ITS1F/ITS4 primer pair to amplify this mycorrhizal type, as it has been used as such in previous studies (Chen and Cairney, 2002). However, in this study the ITS1F/ITS4 primer set poorly amplified ericoid mycorrhiza fungi. There are two possible explanations for the skewed amplification. First, many of the fungal species that form ectomycorrhizas are capable of rhizomorphic growth (Agerer, 1995). This type of growth consists of aggregated mycelium ramifying long distances throughout the soil matrix and would provide much higher levels of biomass to be sampled from the soil. This should increase the possibility of DNA recovery, providing a better template for PCR amplification. ERM and AM fungi do not produce rhizomorphs, and have limited hyphal development into the soil (Schuepp et al., 1987). It is possible that the extent of ERM and AM hyphal development into the soil matrix is too restricted to be adequately sampled by this type of protocol. Second, the ITS1F/ITS4 primers may be biased towards ectomycorrhizae. The ITS1F primer was initially thought to be slightly

biased towards basidiomycetes (Gardes and Bruns, 1993) but this possibility hasn't been thoroughly tested nor has it been a barrier to its utilization in a wide array of studies of non-basidiomycete fungi, including ericoid mycorrhizae (Chen and Cairney, 2002). While ERM fungal DNA was almost certainly abundant in the sampling plots due to the high concentration of rhododendron thickets, few samples returned a positive for the ERM DNA.

Careful testing of the primer sets is required before their further utilization in mycorrhiza diversity studies, such as this one, that addresses more than the basidiomycete community. It is also likely that extensive root sampling for AM and ERM fungi will be needed in addition to soil-based sampling. The root tip DNA analysis in this study wasn't extensive enough to adequately sample the AM and ERM diversity on these plots; this sampling was only meant as a comparison of root and soil based analysis. However, it is interesting to note that root tip DNA recovered from roots sampled from the soil horizons resulted in approximately 15% of the number of mycorrhizal species than the soil based extractions. This gives an indication that many more species of mycorrhizae may inhabit a soil than is measured from root-based analysis. The sampling of plant root tips could lead to the acquisition of only the most dominant mycorrhizal types, while the soil based analysis will include any species within that soil. It is also possible that root sampling provides a temporal snapshot of the root based mycorrhizal community, while other species could be dormant, or saprotrophic within the soil matrix during part of a year.

Cloning and Sequencing:

We utilized 30 clones per sample based upon the work of Landewert et al., (2003), who determined that this sampling level enabled them to detect the top five percent of ectomycorrhizal species in glacial till soil. We initially assessed the feasibility of larger clone sampling, with tests of 50 and 100 clones per sample. However, like Landeweert et al., (2003)

this more extensive sampling resulted in sequence duplications, vector sequences, and non-mycorrhizal fungi. The more extensive clone sampling did not result in significantly greater numbers of useful sequences, and 30 clones per sample threshold may be an appropriate sampling intensity for other soils as well. However, further testing in a variety of soil types is necessary before any methodological generalizations are made.

Mycorrhizal Type Distribution/Synthesis:

ERM fungal DNA was extracted from the O and A horizons, with O horizon recoveries comprising 67% of the ERM total. Extracted ERM DNA had high similarity to three Genbank ERM entries, *Hymenoscyphus ericae, Hymenoscyphus sp*., and an unknown mycorrhizal ascomycete recovered from *Rhododendron maximum*. ECM fungi occurred in the O (8%) and B (16%) soil horizons, but principally within the A horizon (75%). ECM DNA recovered from these soil horizons had high similarities to one of 12 ECM fungal sequences in the Genbank database. AM fungal DNA was extracted almost exclusively (83%) from the B soil horizon. The sequences matched well with three AM species, *Endogone pisiformis, Acaulospora sp.*, and *Glomus* species.

There are clear differences between the soil-based mycorrhizal fungal hyphal distribution and the root-based mycorrhizal fungal distribution (Table 2.3 and Table 2.4). First, while 18 different mycorrhizal fungal sequences were recovered from the soil samples, only 8 different sequences were recovered from the root tip samples, 5 of which matched soil sample sequences. This result might indicate that the soil based sampling is recovering DNA sequences from spores and dead hyphae and is not representative of just the current mycorrhizal fungal community but of the historical fungal community as well. This result might also indicate that there are many more mycorrhizal fungi within a soil community than are typically recovered from root samples.

It is possible that these fungi are only sparsely colonizing root tips, if at all, and may rely on saprotrophic abilities to persist in the soil.

For all three mycorrhizal types the majority of DNA recoveries from root tips were from the A horizon. This distribution of colonization points is different than from hyphal distribution. For example, AM fungi from root tips were recovered principally from A horizon roots, while their soil-based distribution was clustered within the B horizon. This suggests that the AM hyphal development is greater within the B horizon, despite the possibility of greater colonization rates within the A horizon. Due to the nature of the DNA extraction/sequencing technique, it is impossible to tell whether actual biomass of AM fungi is greater within the B horizon soil compared to the A horizon root tips, or if this result is due to protocol bias due to differences in the substrate from which the soil and root samples were recovered. However, the reported functional capacity of AM fungi to utilize the inorganic substrates may be affecting their hyphal development within this soil. ECM and ERM root-based distributions are also different from their soil-based distribution. ECM fungi were recovered from the O horizon root samples to a much greater extent (31%) than from O horizon soil samples (8%). Likewise, ERM root recoveries were principally from the A horizon (63%), while soil-based recoveries were mainly from the O horizon (67%). Some of these differences could be explained by the difficulty encountered separating the O and A soil horizons. It is possible that in some samples material between the O and A horizons were exchanged. It is also possible that while ECM root-based DNA recoveries were proportionally greater within the O horizon than soil recoveries, the ECM hyphae might preferentially develop down into the A horizon and proliferate. The discrepancy in ERM distribution is more difficult to assess. It is possible that root sampling was biased towards recoveries within the A horizon, and that the very fine “hair roots” typical of ERM hosts were present to a greater extent within the O horizon, but were not recovered.

Rhododendron roots can be extremely fine, and typically proliferate around organic matter (pers. Obs.), therefore, the likelihood of missing these fine roots within the O horizon is high.

While this study wasn't designed to determine factors influencing mycorrhizal fungal distribution between soil horizons, it is possible that several soil parameters are involved.

Soil parameters responsible for the change in vertical distribution of mycorrhizal types may include pH (Danielson and Visser, 1989), soil moisture (Bååth and Söderström, 1982), soil structure and temperature (Kernaghan and Harper, 2001), O_2 and CO_2 levels (Bruns, 1995), and mineral and organic nutrient content (Read, 1993). The impact that different levels of organic and inorganic nutrients may have on mycorrhizal community structure has received the most attention. Read (1993, 1991a,b) has written extensively on the landscape level distributions of ERM, ECM and AM fungi, and how the forms of the available nutrients play a key role in this distribution. ERM, ECM, and AM fungi each have unique capabilities for accessing organic and mineral nutrient pools. ERM and ECM fungi possess proteolytic capabilities (Bending and Read, 1996; Tibbet et al. 1999). ERM fungi have demonstrated high activities of cellulase while some ECM produce an enzyme of slightly lower activity (Colpaert and Van Leare, 1996). The ability to use organic N complexed to polyphenols as a nutrient source has been demonstrated in ECM, as well as ERM (Colpaert and Van Leare, 1996). All three mycorrhizal types can acquire P from phytate and inositol phosphates via the production of phosphatases (Mitchell and Read, 1981; Dighton, 1991; Joner and Johansen, 2000). Therefore, each mycorrhizal type produces a suite of enzymes that makes it most efficient on specific substrates.

The soil we studied was gathered from plots that included plant hosts of all three mycorrhizal types. However, an important factor affecting this soil was the presence of rhododendron thickets that produced a litter layer of recalcitrant sclerophyllous foliage. Sclerophyllous foliage typically has a high carbon:nitrogen ratio, and is high in tannins which

enhance the development of protein-tannin complexes, decreases rates of N mineralization (Silvapalen, 1982), and reduces soil pH (Vance et al., 1986). The presence of this type of recalcitrant litter likely played a role in the vertical distribution of the three mycorrhizal types.

Our data indicates that ERM fungi were the most prevalent mycorrhizal type in the O soil horizon. Given the abilities of ERM to utilize a variety of organic substrates as nutrient sources, it is possible that the organic nature of the O soil horizon was a determining factor in ERM distribution.

ECM fungi occurred throughout the soil profile, but were preferentially distributed within the A horizon. While ECM have demonstrated much variability in vertical distribution (Goodman and Trofymow, 1998), many studies have indicated that most hyphal development occurs within the fermentation horizon, the boundary between the O and A soil horizons (Read, 1990). However, few occurrences of ECM fungi were recovered from the O horizon, indicating that the majority of ECM might have been restricted to the lower A horizon. This distribution might indicate that ECM was competitively excluded from the lower O horizon by the presence of ERM fungi. A negative growth response by ECM root tips when in the presence of rhododendron has been noted by Nilsen et al., (1999), who suggests that allelopathy might be a cause of this reduction.

AM were recovered almost exclusively from the B soil horizon. AM fungi lack the capability to produce a variety of enzymes enabling the fungi to utilize organic N sources and only minimal ability to utilize organic P (Read and Perez-Moreno, 2003). Therefore, it is possible that the localization of AM in the lower soil horizon is due to the distribution of organic substrates in the upper soil horizons.

Further studies aimed at better defining the vertical and horizontal distributions of these three mycorrhizal types and the factors that impact them are needed. The distribution of

mycorrhizal types over organic and inorganic nitrogen and phosphorus gradients is an obvious next step. However, the effects of soil moisture, texture, mineralization rates, pH and other soil properties need study as well. Also, a better understanding of how different mycorrhizal types compete against each other and how different species of the same mycorrhizal type compete are necessary to understand their dynamics in communities such as the one studied here.

The molecular tools employed in this study can greatly improve our ability to map mycorrhizal hyphal distributions. However, there exists potential for bias with each step. Soil sampling procedures need to be tested to verify that the mycorrhizal community is being accurately sampled both on a horizontal and vertical plane. Soil DNA extraction procedures need to be optimized and tested in order to assure that the DNA recovered isn't biased towards certain groups of mycorrhizal fungi. Further primer development is needed to assure PCR amplifies all fungi of interest. Lastly, clone sampling needs further testing to determine the appropriate number of clones to evaluate for different soil types.

SUMMARY

A fast, simple, and precise methodology for mycorrhizal fungal community diversity analysis is necessary before research can be conducted over larger scales of time and space. The utilization of soil based DNA testing is a clear contender for fulfilling this technological need. In this study, we presented results of several DNA extraction techniques. The result of this testing indicates that a commercially produced kit for DNA extraction from soil (QbioGene Inc.) outperformed the other protocols. We also demonstrate that, for studies aimed at multiple types of mycorrhizae, further primer development is required to achieve adequate representation of AM and ERM fungi.

Despite these methodological difficulties, the data we obtained indicates that the three major types of mycorrhizae (ECM, AM, and ERM) are unequally distributed between soil horizons. AM fungi were principally found within the B soil horizon, ERM fungi were found principally in the O horizons with some occurrence in the A horizon, while ECM fungi occurred in all soil horizons, though predominately the A horizon. This distribution may be a result of "niche separation" as discussed by Dickie et al., (2003), since each of the three mycorrhizal types has unique enzymatic capacities to utilize organic substrates.

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CHAPTER 3

THE VERTICAL DISTRIBUTIONS OF ERICOID, ECTO-, AND ARBUSCULAR MYCORRHIZAL FUNGI AND THEIR CORRELATION TO SOIL N AND P DISTRIBUTION[1]

[1] Rachel, G.C. and R.L. Hendrick. To be submitted to *Mycorrhiza*

ABSTRACT

Understanding the factors affecting the spatial distribution of mycorrhizal fungi in soils is critical to understanding their impacts on forest communities. However, details about what soil variables are most important in mycorrhizal fungal spatial distribution are few. We investigated the vertical distribution, of ericoid (ERM), ecto- (ECM), and arbuscular (AM) mycorrhizal fungi in a soil of a mixed species forest at the Coweeta Hydrologic Lab, Otto, N.C., and attempted to determine if this spatial distribution was correlated with N and P fraction distribution. The soil contained host plant roots of all three mycorrhizal fungi, *Rhododendron maximum* (ERM), *Tsuga canadensis* (ECM), and *Liriodendron tulipifera* (AM). We utilized PCR, cloning and DNA sequencing to determine mycorrhizal fungal distribution. Soil was retrieved from three soil horizons (O,A,B) and DNA extracted from the soil was PCR amplified, cloned and sequenced. Sequences were identified using BLAST searches and by utilizing Maximum-Likelihood analysis. The correlation between mycorrhizal fungal distribution and N and P fraction distribution were tested using Principal Component Analysis. Our results indicate that the three mycorrhizal types are differentially distributed throughout the soil horizons. ERM fungi occurred predominately within the O soil horizons and AM fungi occurred mainly in B soil horizons. The small majority of ECM fungi were located within the A soil horizon and ECM fungi were found in the O and B a well. ERM fungi were correlated with high concentrations of inorganic N and organic N and P. AM fungi were negatively correlated with inorganic and organic N, while ECM occurred throughout the N and P fraction distribution. We hypothesize that this fungal distribution relates to the capacity of each fungal type to utilize various soil substrates as nutrient sources.

Introduction

Mycorrhizal fungi are critical components of almost every terrestrial ecosystem (Smith and Read, 1997), and there are thousands of fungal species capable of forming mycorrhizas. These multitudes of species comprise several different classes of mycorrhizae, three of which are predominant; ericoid (ERM), ecto- (ECM), arbuscular mycorrhizae (AM). Each mycorrhizal type differs in its ability to acquire soil resources and transfer them to its symbiotic plant partner (Read and Perez-Moreno, 2003). These differences are important, as the effective functioning of a specific symbiosis could influence both mycorrhizal and plant community composition(Heijden et al., 1998a,b). Critical factors influencing the capacity of mycorrhizas to acquire nutrients are their distribution throughout the soil profile and the soil factors that control this distribution, such as organic and inorganic nitrogen and phosphorus. However, relatively little is known about either the community structure and temporal dynamics of mycorrhizal fungal communities or the factors which influence them; relevant studies are few in number (e.g. Dahlberg, 2001).

Of the three dominant mycorrhizal types, the community ecology of ECM fungi has been the most researched (Jones et al., 2003, Dahlberg, 2001, Horton and Bruns, 2001). ECM fungal community assessments based on root tip studies, as opposed to hyphal distribution, have shown that ECM generally possess aggregated distributions (Gardes and Bruns, 1996). Aggregate distributions are thought to occur because the fungi are impacted by host plant distribution (Molina et al., 1992) as well the heterogeneous nature of soil properties (Kernaghan and Harper, 2001; Dickie et al., 2002). For example, ECM root tip vertical distributions are affected by water availability (Taylor and Bruns, 1999), as well as soil structure and chemical composition (Stendell et al., 1999). Tederosoo et al., (2003) demonstrated that monophyletic groups of ECM fungi differed in their preference for soil substrates, a difference that they attribute to the fungi's

differing degradative enzymatic capabilities. Jonsson et al., (2000) found that 90% of the ectomycorrhizas were located within the organic soil layer of a *Picea abies* forest, while a few specific species, such as *Cenococcum*, were evenly distributed between mineral and organic soil layers. Goodman and Trofymow (1998) showed that ectomycorrhizas occur on a wide range of substrates, from decaying wood and forest floor, to mineral soil; many individual species are limited to a single substrate type. They also compared ectomycorrhizal root tip distribution to the abundance of mineralizable N and extractable P and found that several ECM types were directly affected by the N and P content. Some ECM fungal species, however, are uniformly distributed (Gardes and Bruns, 1996, Stendell et al., 1999) and occur throughout a forest stand. Thus, there are varying types of spatial distributions for different ECM fungal species. These data, however, must be considered in light of the fact that most ECM fungal community studies have focused on fruiting bodies or root tips to determine population distribution, and that the corresponding distribution of hyphae (those structures responsible for most uptake and enzyme production) remains unknown (Horton and Bruns, 2001).

The most prevalent type of mycorrhizae are AM; two-thirds of plants are capable of forming mycorrhizae of this type (Fitter and Moyerson, 1996). It is generally thought that AM fungi are more uniformly distributed in comparison to ECM fungi, and that AM fungi are less host plant specific than ECM fungi (Smith and Read, 1997). However, other studies contradict these theories. Several studies have demonstrated an aggregate distribution for AM fungi (Klironomos et al., 1993, Friese and Koske, 1991), which may be influenced by edaphic factors such as soil moisture (Anderson et al., 1986) or pH (Coughlan et al., 2000). Husband et al.(2002) and Helgason et al. (2002) suggest that there is more host specificity and site preference among AM fungi than previously thought. AM fungal community studies have typically been carried out using spore counts to represent community structure. This procedure

may lead to confusion in AM fungal community analysis, as many factors affect spore production and diversity (Eom et al., 2000, Morton et al., 1995).

Much less information is available about the community composition and dynamics of ERM fungi. ERM form on plants of the Ericaceae and many ERM fungi possess the ability to degrade recalcitrant organic substrates such as protein-tannin complexes and lignin (Read and Perez-Moreno, 2003), thereby acquiring otherwise inaccessible forms of N and P, (Cairney, 2000). The number of ERM fungal species is unknown (Perotto et al. 2002) and the relatedness of the known species is debatable (Vralstad et al., 2000; Sharples et al., 2000a), making the study of ERM fungal community dynamics even more problematical. We could find no study concerning the stand based distribution and dynamics of ericoid mycorrhizae.

We sought to determine if the distribution of the three predominant mycorrhizal types along a vertical soil profile in a mixed hardwood forest corresponded to the nitrogen and phosphorus fractions within each soil horizon. We sampled soils from a forested stand comprising all three major types of mycorrhizae (ectomycorrhizae, arbuscular mycorrhiza, and ericoid mycorrhizae). Using DNA extracted from three soil horizons, along with measurements of N and P in each horizon, we attempted to determine if the distribution of each type of mycorrhizae corresponds to the type and amount of N and P. Dickie et al. (2002) hypothesized that the vertical distributions of various ECM species in their study might be due to niche separation of the ECM based on functional differences. These functional differences might be based on host specificity, tree age or another of the various morphological or biochemical differences presented above.

Our hypothesis for the distribution of the different mycorrhizal types was that the putative ability of each type to acquire nutrients from different substrates would be the critical factor in their pattern of soil distribution. Given the documented abilities of each type to acquire N and P

from different organic and inorganic sources (Read and Perez-Moreno, 2003), we hypothesize that ERM will occur predominately in the O soil horizon, ECM will primarily occur in the A horizon, and AM in the B soil horizon.

METHODS:

Site Characteristics:

The soil samples utilized in the study were collected at the Coweeta Hydrologic Laboratory in Otto, North Carolina (35°02'44"N, 83°27'09"W). The sampling plots were located in a cove forest with a Humic Hapludult soil. The principal vegetation includes a *Liriodendron tulipifera* overstory, *Tsuga canedensis* in the sub-dominant canopy to understory position, with *Rhododendron maxima* understory. *L.tulipifera* is associated with arbuscular mycorrhizae (AM), *T. canadensis* forms ectomycorrhizae (ECM), and *R. maxima* form ericoid mycorrhizae (ERM). Four sampling plots, each $25m^2$, were located within the cove forest areas. Within each plot, three subplots were randomly located. Within each subplot soil samples were collected from three depths. The sampling methodology involved digging a soil pit, $900cm^2$ in area, through the B-horizon and collecting the O, A, and B horizons. Soil samples were stored in plastic bags and frozen with dry ice for the trip back to the laboratory.

Sample preparation:

In preparation for DNA extraction, soil samples were cleaned of root fragments by hand, air dried for 3 days, ground with a mortar and pestle, passed through a 125µm sieve, then freeze-dried and stored at -20°C. O horizons samples were also ground with a Wiley mill before sieving. Roots removed from the soil samples were freeze-dried and stored for subsequent DNA extraction and analysis.

DNA Extraction from Soils:

DNA extraction from soils involved the use of QbioGene's FAST DNA Spin Kit for soils (QbioGene Inc., 2003). The kit incorporates bead beating in detergent, followed by guanidine isothiocyanate purification and ethanol washing.

PCR Amplification of Soil DNA Extracts:

Six different primer combinations were utilized to cover the range of ECM, ERM, and AM fungi likely to be present within the test soils. ITS1F/ITS4 was used to amplify the ECM and ERM taxa. This primer set has been utilized for amplification of ectomycorrhizae and ericoid mycorrhizae (Chen and Cairney, 2002; Gardes and Bruns, 1993). GLOM5.8R, and GIGA5.8R in combination with ITS1F and ARCH1311, ACAU1660, and LETC1670 combined with ITS4, was utilized to amplify the various AM taxa. Redecker (2000) developed these primer sets to amplify AM fungi from root samples. The GLOM5.8R/GIGA5.8R/ITS1F combination amplifies the *Gigasporaceae* and the *G. mosseae/interadices* group while the ARCH1311, ACAU1660, LETC1670, ITS4 combination amplifies the *Acaulosporaceae, Glomus enticatum/clarodium* group, and the *A. germannii* and *G. occultum* groups (Redecker, 2000). The PCR protocol followed that of Redecker (2000).

Analysis of PCR Products:

PCR products from soil DNA extracts were cloned using Invitrogen's TOPO-TA 2.1 cloning kit (Invitrogen, 2004) following the manufacturers instructions. Approximately 30 clones per PCR reaction were collected. The clones were then mini-prepped and the DNA sequenced using an ABI 3700 sequencer with ABI BigDye version 2 or 3 chemistry (Applied Biosystems, 2004).

Sequence Identification:

Sequences were analyzed using the GCG Sequence Analysis software package (Wisconsin Package version 10.2, Genetics Computer Group, 2004). The GelAssemble module was utilized to compare sequences to each other in order to merge duplicates, remove poor sequences, and to trim vector sequences from the fungal sequences. The sequences were then individually viewed and any base pair adjustments that were required to make the base sequence to better match the sequence trace curves were made. BatchBlastX was then utilized to compare the sequences to the National Center for Biotechnology Information (NCBI) database. Those sequences showing close matches to mycorrhizal species were retained and ClustalX was used to align the sequences to each other and their nearest Blast matches as well as a variety of mycorrhizal species. The aligned sequences were then entered into the PAUPSearch/PAUPDisplay module of GCG. Using the maximum-likelihood criterion, a phylogenetic tree was constructed.

Nitrogen Fractionation:

The nitrogen fractionation procedure followed the protocol of Mulvanney (1996a). In brief, 3g of finely ground soil was hydrolyzed by boiling for 12 hours in 6M HCl. The hydrolysate was filtered and utilized in the subsequent fractionations. Six N fractions were determined:

(1) Total Hydroloyzable Nitrogen: Five milliliters of the hydrolysate was digested with H_2SO_4 for 12 hours. The flask of hydrolysate was then connected to the steam distillation apparatus and 10ml of 10M NaOH added to the flask. The mixture was then steam distilled until 35ml of condensate was collected in a beaker containing 5ml of boric acid indicator solution. Ammonium in the condensate was measured by titration with 0.0025M H_2SO_4.

(2) Amino Acid Nitrogen: One milliliter of 0.5M NaOH was added to 5ml of hydrolysate

and boiled for 20 minutes. Five hundred milligrams of citric acid and 100 ml ninhydrin was then added to the flask and the mixture was boiled for 10 minutes. After the flask cooled, 1.25 g of phosphate-borate buffer and 10 ml deionized water were added to the flask. The flask was attached to the steam distillation apparatus and 1 ml of 5M NaOH was added to the flask and steam distillation began. Distillate was captured in a flask containing boric acid indicator solution. Distillation was ceased once the distillate level reached 35 ml. The distillate was titrated with H_2SO_4.

(3) Ammonia Fraction: Ten milliliters of hydrolysate was placed into a flask, 0.07 g of MgO was added, and the mixture steam distilled until the distillate volume reached 20 ml. The distillate was then titrated with H_2SO_4.

(4) Ammonia + Amino Sugar Nitrogen: Ten milliliters of hydrolysate was placed into the distillation flask and 1.25 g phosphate-borate buffer was added. The mixture was distilled and titrated.

(5) Amino Sugar Nitrogen: Calculated as the difference between Ammonia+Sugar fraction and Ammonia N fraction.

(6) Hydrolysable Unknown: Calculated as the difference between total hydrolysable - (amino acid + amino sugar + ammonia).

Nitrate and ammonium were measured using the protocol of Mulvaney (1996b). Briefly, ammonium was measured by placing 20 ml of hydroloysate into the distillation flask and adding 0.2 g of MgO. The mixture was steam distilled and titrated as above. Nitrate was measured immediately after the ammonium measurement by placing 0.2 g Devarda's alloy into the distillation flask, and distilling and titrating as above.

Phosphorus Fractionation:

The phosphorus fractionation procedure follows that of Tiessen and Moir (1993). Nine fractions were recovered using this protocol:

(1) Resin extractable: One half gram of air dried soil was sieved and placed into a 50 ml centrifuge tube. Two resin strips (Bio-Rad AG 1-X8) and 30 ml DI water were added and the tube was shaken overnight at 120 rpm. The resin strips were transferred to new tubes and 20 ml 0.5M HCl was added. After allowing the tubes to degas, they were again shaken overnight. The resin strips were removed and 15 mls of extract was used to determine inorganic P. The extract was pH adjusted to pH7 with 4M NaOH and 0.0025M H_2SO_4 using p-nitrophenol as the indicator. Murphy-Riley reagent was added to each extract and allowed to react for 20 min. The absorbance of the extract at 885 nm was then determined using a Beckman-Coulter DU-64 spectrophotometer. Each run included 7 standards (0, 60, 120, 240, 600, 1200, 2400 ppb) and phosphorus spiked samples (0, 60, 120, 240, 600, 1200, 2400 ppb).

Each of the 9 remaining fractionation measurements below was preceded by the following extraction procedure:

One half gram of air-dried, 125μm sieved soil was placed into a 50 ml centrifuge tube with 30 mls of 0.5M $NaHCO_3$ and shaken overnight. The tubes were centrifuged for 10 min at 3400 rpm, followed by vacuum filtration through Millipore 47μm cellulose acetate/nitrate filters into new 50 ml tubes to obtain the $NaHCO_3$ extract, which was stored at 4°C . Soil remaining on the filter was returned to the original tube, 30 mls of 0.1M NaOH was added and the tubes were again shaken overnight. The tubes were then centrifuged and vacuum filtered to obtain the NaOH extract. Soil remaining on the filter was returned to the original tube and 30 mls 1M HCl added to the tube and shaken overnight. The tubes were centrifuged and filtered to obtain the 1M HCl extract. Ten milliliters of concentrated HCl was then added to the tubes containing the

soil and heated to 80°C for 25 minutes, after which an additional 5 mls of HCl was added and the mixture allowed to sit for 1 hour. The tubes were then centrifuged for 10 minutes and decanted into new tubes to collect the concentrated HCl extract. The soil remaining in the tubes was allowed to dry, at which time 0.1 g of each soil was utilized in a nitric/perchloric acid digest for residual P.

(2) $NaHCO_3$ inorganic P and (3). 0.1M NaOH inorganic P: Six mls of 0.9M H_2SO_4 was added to ten milliliters of the $NaHCO_3$ extract and 1.6 mls 0.9M H_2SO_4 was added to NaOH extracts. After 30 minutes at 4°C the mixtures were centrifuged for 10 minutes at 3400 rpm before decanting. The mixtures were adjusted to pH 7 using 4M NaOH and 0.0025M H_2SO_4 using p-nitrophenol as the indicator, and then Murphy-Riley P was measured as previously described.

(4) NaHCO3 total P, (5) 0.1M NaOH total P, and (6) concentrated HCl total P: One half gram of ammonium persulfate and 10 mls of 0.9M H_2SO_4 was added to 5 mls of the $NaHCO_3$ extract; 0.6 g ammonium persulfate and 10 mls 0.9M H_2SO_4 was added to NaOH extracts; and 0.4 g and 10 mls of DI water was added to the concentrated HCl extracts. The extracts were then autoclaved for 90 minutes. The pH was then adjusted and Murphy-Riley P measured.

(7) 1M HCl inorganic P, (8) concentrated HCl inorganic P, and (9) residual P: Fifteen milliliters of each extract was transferred to a 50 ml tube. The pH was adjusted and Murphy-Riley P measured.

Analysis of mycorrhizal type distribution:

The correlations between mycorrhizal type distribution and the N and P fraction distributions were quantified using principal component analysis (PCA) with the PRINCOMP procedure in SAS 8.2 (SAS, 2003). Initially, all N and P factors were utilized. However, including each term reduced the percentage of the variability accounted for by each principal

component, and didn't reduce the number of dimensions of data. Therefore, each organic and inorganic N and P measurement was pooled.

RESULTS:

Sequencing Results:

From the 48 soil samples collected, 1440 clones were processed, producing 128 unique fungal sequences that were recovered and analyzed utilizing Genbank's Blast utility. Of these, 42 of the sequences were possibly mycorrhizal fungi (Table 3.1). The phylogenetic tree created with the sequences (Figure 3.1), their closest Blast match, and several known mycorrhizal fungal sequences revealed that most of the sampled sequences fall clearly within a mycorrhizal group. A few sequences, however, fall outside a known mycorrhizal group, and were not further analyzed. The phylogenetic tree enabled us to limit the 42 sequences to 18 groups or operational taxonomic units (OTU)(Table 3.2).

OTU Distribution:

Fisher's Exact Test (Fisher, 1973) indicates that the mycorrhizal type distribution between soil horizons is non-random (p-value < 0.0001) . There were 112 instances of recovering mycorrhizal DNA from the 48 samples. Of these 112 instances 97 were ectomycorrhizal DNA, 6 were arbuscular mycorrhizal DNA, and 9 were ericoid mycorrhizal DNA. The majority of ericoid mycorrhizae (67%) were recovered from the O soil horizon, 75% of ectomycorrhizae

Table 3.1 - Nearest BLAST matches for each unique sequence obtained from soil DNA extracts. DNA Extracts were obtained from soil collected from plots located within the Coweeta Hydrologic Laboratory, Otto, N.C. during August 2002. Plots were within cove forest sites populated by *Liriodendron tulipifera, Tsuga canadensis* and *Rhododendron maximum*. DNA extraction involved the use of the FAST-SPIN kit for Soil (QbioGene, Inc. 2002).

Soil Retreived Sequence #	Closest Blast Match	Ascension no.	e-value	percent match
1	*Cortinariaceae* mycorrhizal sp.	AF430290	2.00E-87	95
2	*Cortinariaceae* mycorrhizal sp.	AF430290	3.00E-92	99
3	*Cortinariaceae* mycorrhizal sp.	AF430290	2.00E-89	97
4	*Cortinariaceae* mycorrhizal sp.	AF430290	5.00E-74	91
5	*Clavulinaceae sp.*	CSP 534708	-117	99
6	*Clavulinaceae sp.*	CSP 534708	-135	100
7	*Clavulinaceae sp.*	CSP 534708	-142	100
8	*Clavulinaceae sp.*	CSP 534708	-148	100
9	*Tomentella sp.*	TOM534916	-124	100
10	*Tomentella sp.*	TOM534916	-115	99
11	*Russula virescens*	AY061728	-103	98
12	*Russula virescens*	AY061728	-127	99
13	*Russula virescens*	AY061728	-136	97
14	ascomycete leaf litter fungi	AF502802	-121	100
15	unidentified ascomycete	FA8279453	3.00E-79	95
16	*Amanita vaginata*	AB015693	3.00E-85	96
17	*Amanita vaginata*	AB015693	2.00E-87	97
18	*Amanita vaginata*	AB015693	2.00E-92	99
19	*Amanita vaginata*	AB015693	4.00E-97	99
20	*Clavicorona taxophila*	AF033344	2.00E-83	96
21	*Clavicorona taxophila*	AF033344	3.00E-73	95
22	*Endogone pisiformis*	AF006511	-59	99
23	*Endogone pisiformis*	AF006511	-23	95
24	*Trichloma myomyces*	AF287841	-126	100
25	*Panus rudis*	PRU59086	8.00E-61	98
26	*Omphalina velutipes*	OVU66455	2.00E-55	98
27	*Inocybe relicina*	AY038324	5.00E-19	94
28	Homobasidiomycete sp.	HSP534714	2.00E-51	97
29	*Hebeloma* mycorrhizal isolate	AF432845	9.00E-85	95
30	*Serpula lacrymans*	AJ536023	0	100
31	*Russula atropurpurea*	AF418618	0	100

Table 1 – cont.

32	*Suillus bovinus*	AJ419215	0	100
33	*Tricholoma sp.*	AY097046	8.00E-70	99
34	*Pleurotus pulmonaris*	PPU60648	5.00E-74	97
35	*Phaeotellus griseopallidus*	PGU66436	8.00E-70	98
36	*Acaulospora sp.*	ASP541799	8.00E-82	97
37	*Acaulospora sp.*	ASP541799	2.00E-67	95
38	*Tomentella sp.*	TSU83481	0.006	87
39	*Glomus sp.*	AY035654	2.00E-070	97
40	*Hymenoscyphus ericae*	AY046963	-56	96
41	Mycorrhizal ascomycete of *Rhododendron*	AB089667	4.00E-88	99
42	*Hymenoscyphus sp.*	AF252835	5.00E-74	90

Figure 3.1 – Phylogenetic tree created from the DNA sequences extracted from soil samples. 2a) diagram representing the entire phylogenetic tree. 2b,c,d) Exploded views of each phylogenetic tree section as shown in figure 2a. The unrooted phylogenetic tree was created using the maximum parsimony method with the PAUP/GCG software.

Figure 1a

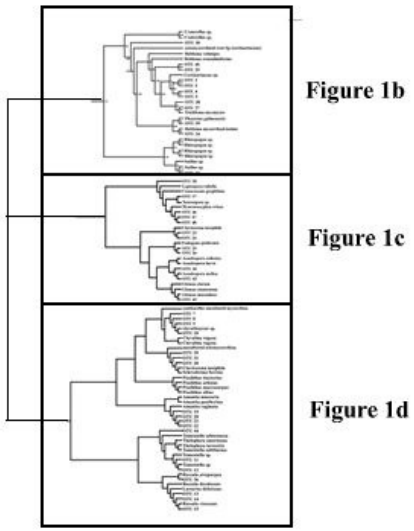
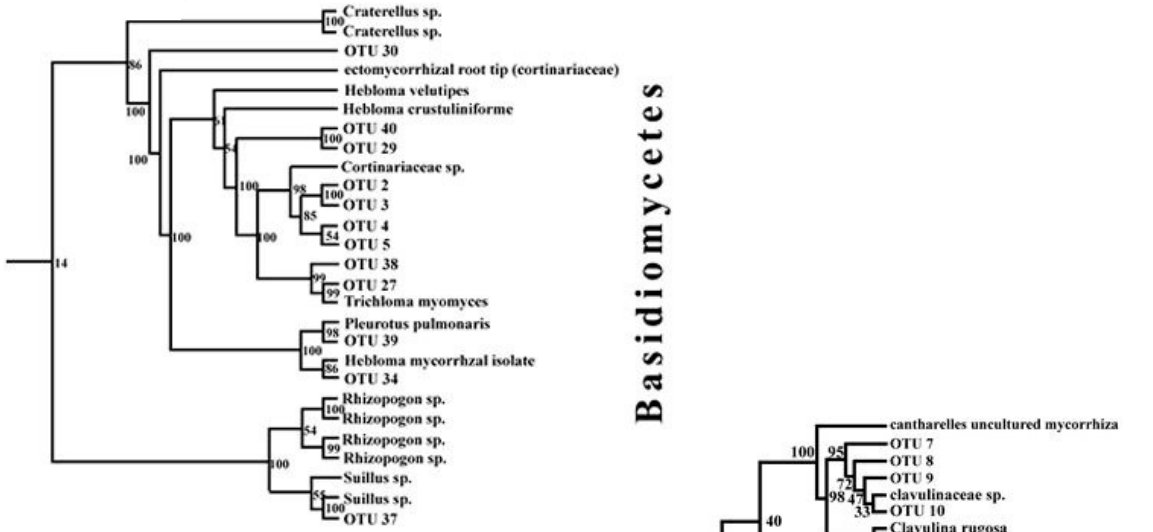


Figure 1b



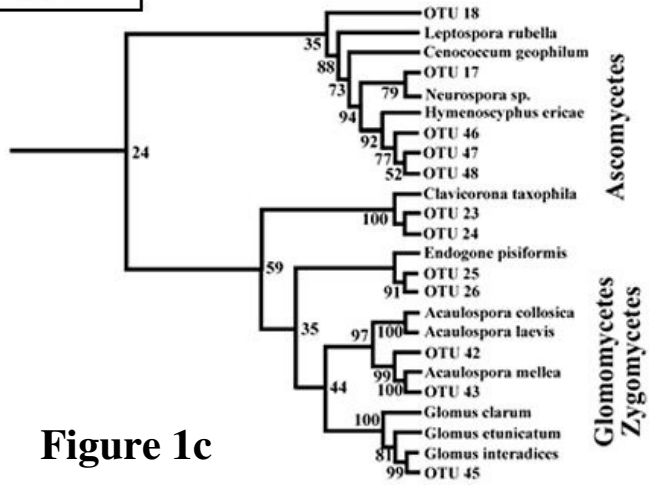


Figure 1c

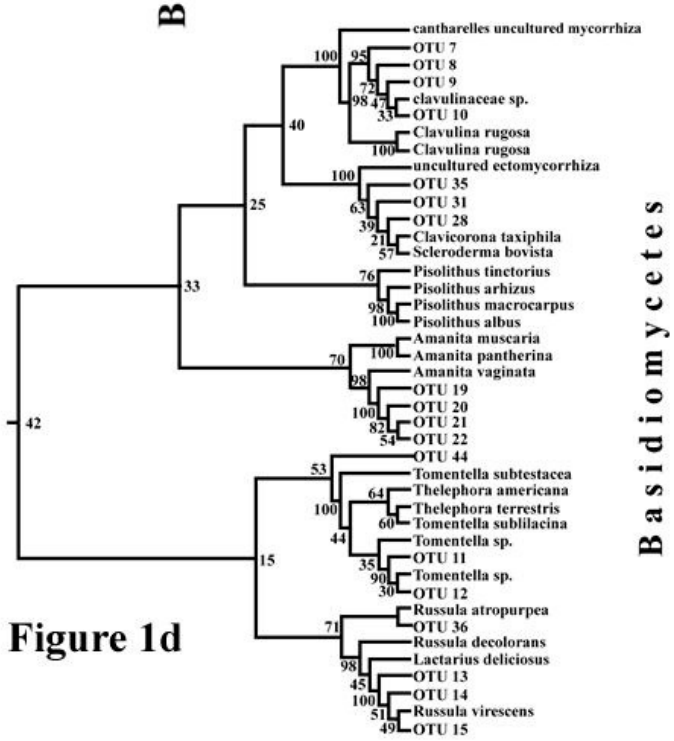


Figure 1d

Table 3.2 -Final OTU identities and mycorrhizal type groupings for the soil derived mycorrhizal DNA. DNA Extracts were obtained from soil collected from plots located within the Coweeta Hydrologic Laboratory, Otto, N.C. during August 2002. Plots were within cove forest sites populated by *Liriodendron tulipifera, Tsuga canadensis* and *Rhododendron maximum*. DNA extraction involved the use of the FAST-SPIN kit for Soil (QbioGene, Inc. 2002).

Soil OTU #	Identity	Mycorrhizal Type
1	*Cortinariaceae* mycorrhizal sp.	ECM
2	*Clavulinaceae sp.*	ECM
3	*Tomentella sp.*	ECM
4	*Russula virescens*	ECM
5	*Amanita vaginata*	ECM
6	*Clavicorona taxophila*	ECM
7	*Endogone pisiformis*	AM
8	*Trichloma myomyces*	ECM
9	*Inocybe relicina*	ECM
10	*Hebeloma* mycorrhizal isolate	ECM
11	*Russula atropurpurea*	ECM
12	*Suillus sp.*	ECM
13	*Tricholoma sp.*	ECM
14	*Acaulospora sp.*	AM
15	*Glomus sp.*	AM
16	*Hymenoscyphus ericae*	ERM
17	Mycorrhizal ascomycete of *Rhododendron*	ERM
18	*Hymenoscyphus sp.*	ERM

were recovered from the A horizon, while 83% of the arbuscular mycorrhizae were recovered from the B horizon (Table 3.3).

Nitrogen Fractionation:

Nitrogen fractions were distributed as expected throughout the soil profile. Organic nitrogen fractions were greater in the O horizon and decreased into the B-horizon (Figure 3.2). Mineral nitrogen distribution was similar in that the nitrate N was greater in the O horizon and decreased into the B-horizon. There was no clear differentiation between ammonium N levels in each horizon (Figure 3.3).

Phosphorus Fractionation:

Phosphorus abundance varied with depth. The bicarbonate inorganic P (Pi) fraction increased from the O horizon to the B horizon, the NaOH Pi, 1M HCl Pi, and the HCl Pi fractions all remained steady through each horizon (Figure 3.4). The other P fractions (bicarbonate organic P (Po), NaOH Po, HCl Po, residual P) all decreased with depth.

Comparison of DNA distribution with N and P Fractions:

There were three significant principal components found in the analysis, representing 93% of the variation in the data. Principal component 1 was predominantly inorganic nitrogen and organic P, principal component 2 included organic N and inorganic P, principal component 3 was organic P and inorganic P (Table 3.4). Each type of mycorrhizal fungi clustered differently in the principal component analysis. ERM fungi were the most unique and occurred in areas where inorganic N and P and organic N were higher, generally within the O horizon layers. ERM fungal distribution in the PCA plots were not related to organic P distribution (Figure 3.5a,b,c). AM fungi plotted principally in the zones that correspond to lower Ni and No and were neutral for Pi and Po. ECM fungi were essentially uniformly distributed throughout the N and P ranges. There were no specific zones on the PCA plots where ECM fungi did not occur.

Table 3.3 - Number of recoveries of each mycorrhizal type within the three soil horizons (AM-arbuscular mycorrhiza, ECM-ectomycorrhiza, ERM-ericoid mycorrhiza). Numbers in parentheses indicate percentage of each mycorrhizal type found in each soil horizon.

	Soil Horizon			
Mycorrhizal Type	O	A	B	Total No.
AM	0 (0)	1 (16)	5 (83)	6
ECM	8 (8)	73 (75)	16 (16)	97
ERM	6 (67)	3 (33)	0 (0)	9

Figure 3.2– Organic nitrogen fractions. Soil samples were collected during the summer of 2001 from plot located in the Coweeta Hydrologic Laboratory, Otto, N.C. The plots were located within cove hardwood areas. The fraction definitions are: tot hyd = total hydrolysable nitrogen; aa = amino acid nitrogen; amm = ammonia nitrogen; amsug = amino sugar nitrogen; unk hyd = unknown hydrolysed nitrogen.

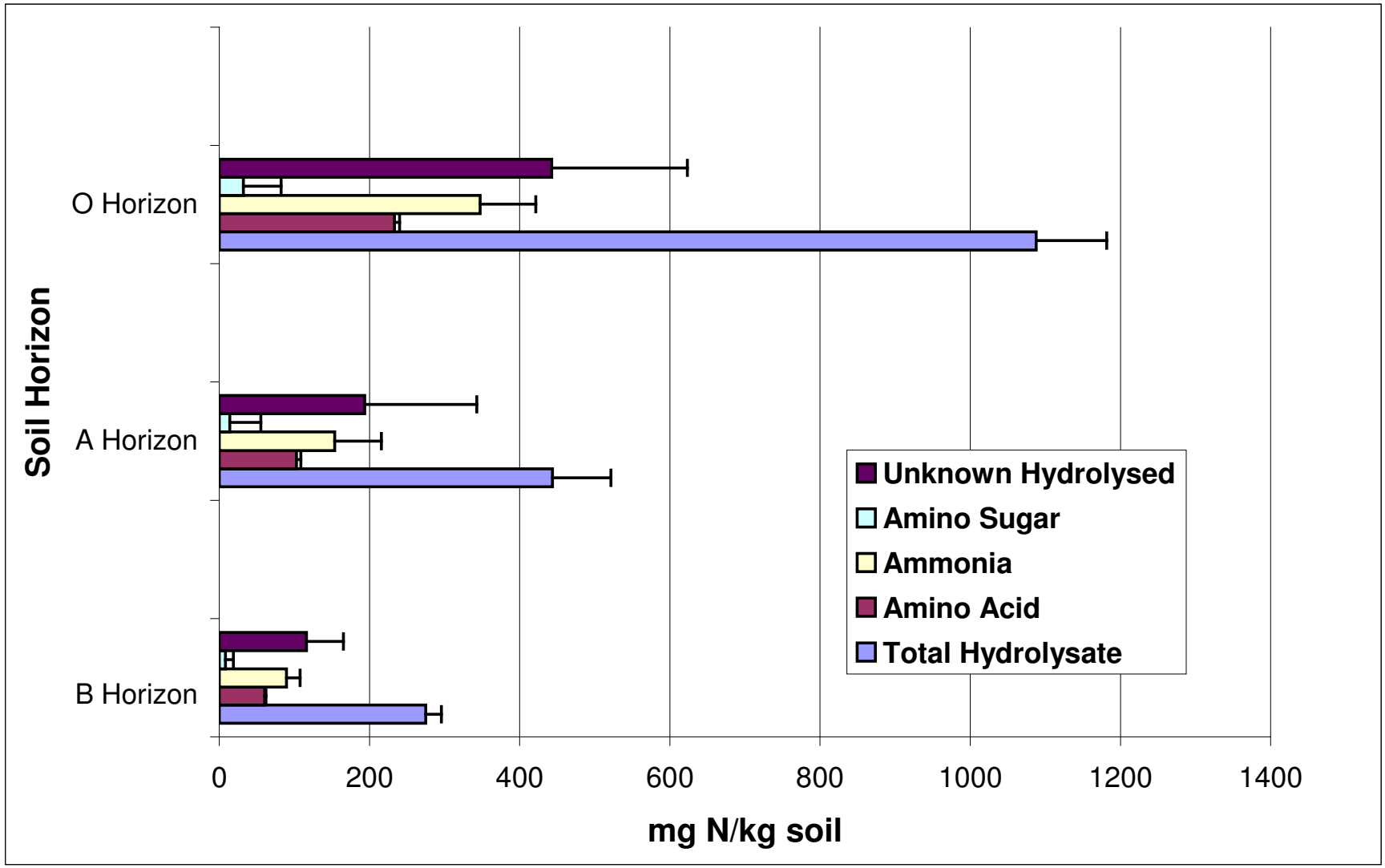

Soil Horizon
O Horizon
A Horizon
B Horizon
0
200
400
600
800
1000
1200
1400
mg N/kg soil
Unknown Hydrolysed
Amino Sugar
Ammonia
Amino Acid
Total Hydrolysate

Figure 3.3 – Nitrate and ammonium measurements from the O, A, and B soil horizons. Soil samples were collected during the summer of 2001 from plot located in the Coweeta Hydrologic Laboratory, Otto, N.C. The plots were located within cove hardwood areas.

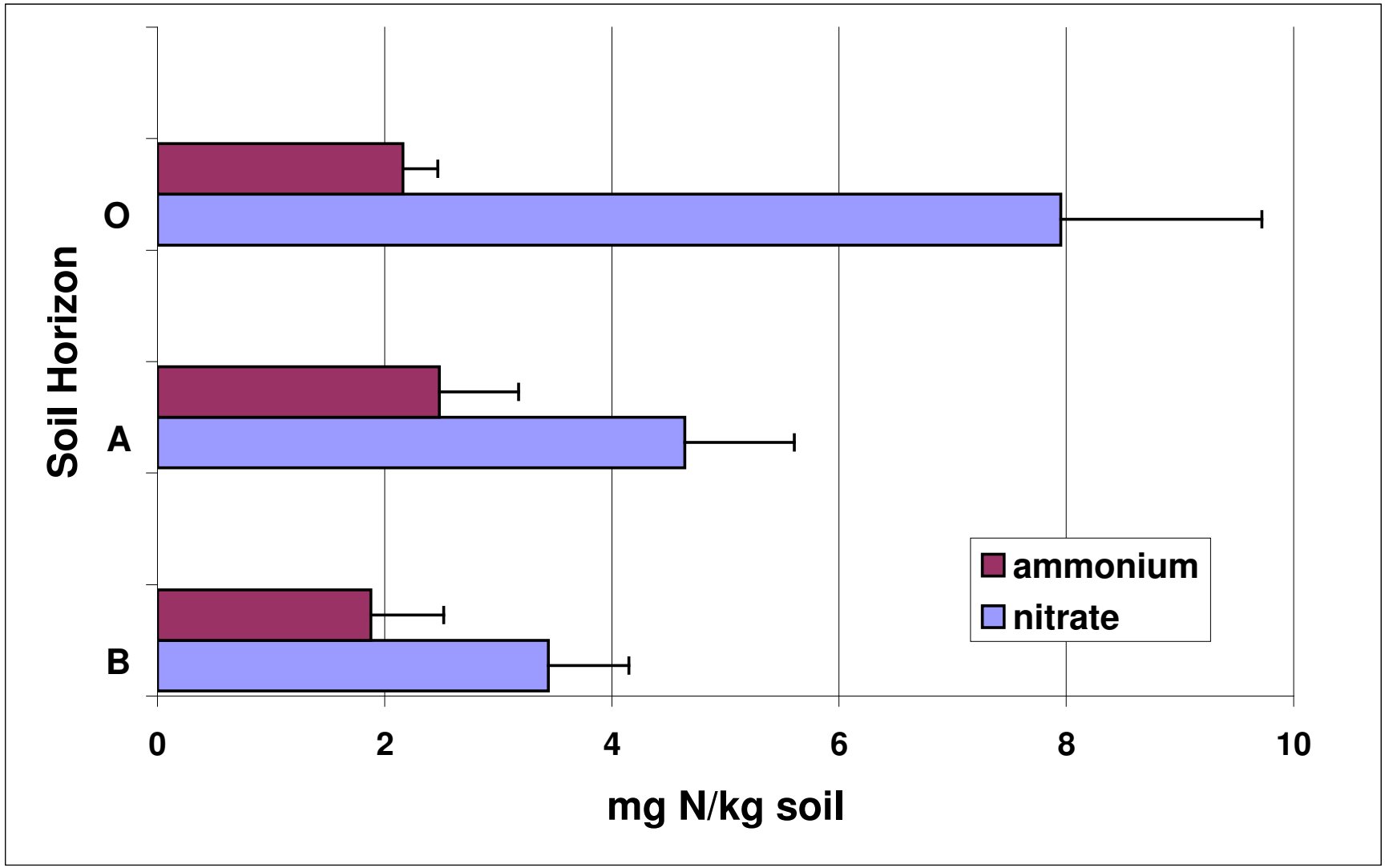
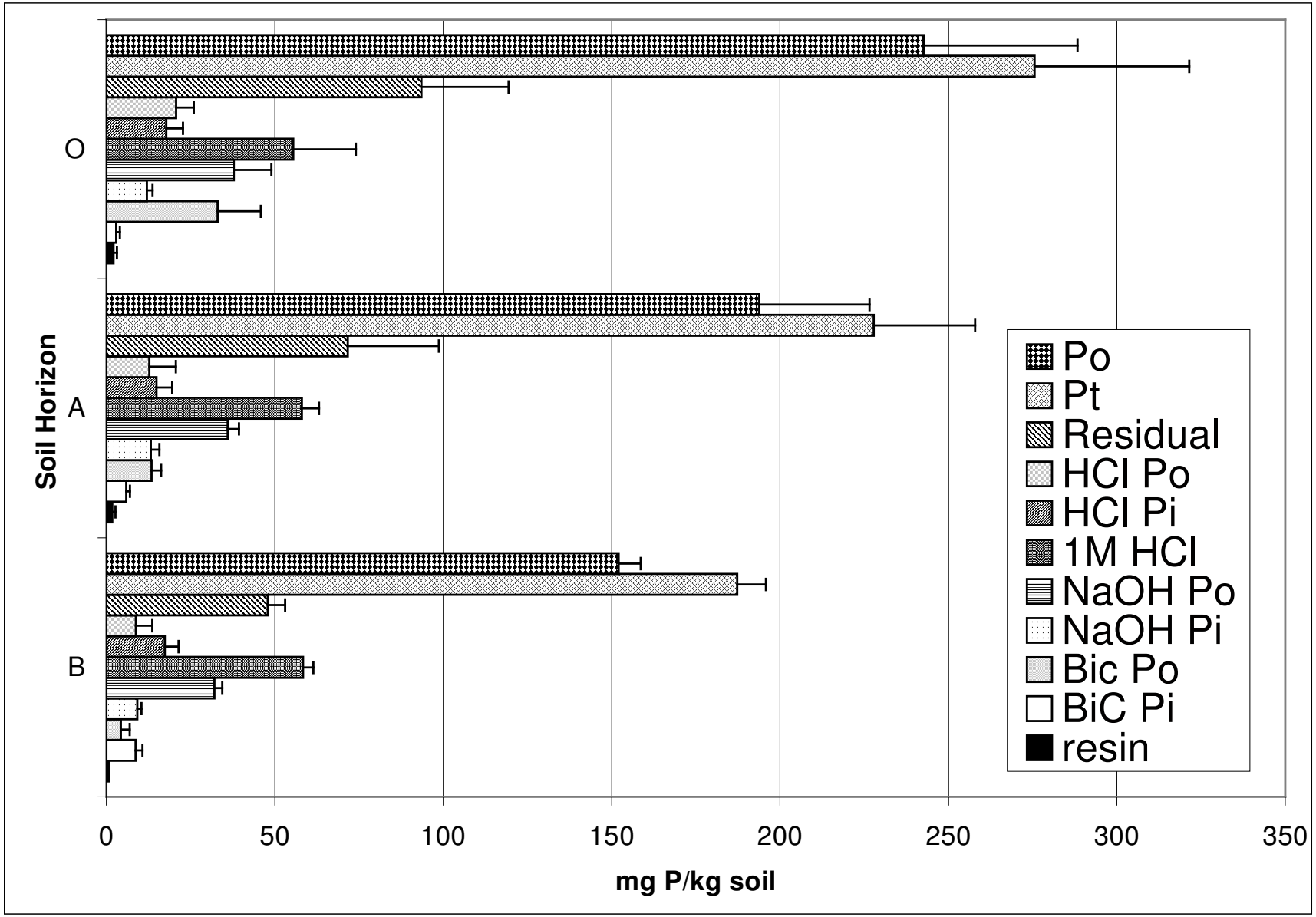

Soil Horizon
O
A
B
0
2
4
6
8
10
mg N/kg soil
ammonium
nitrate

Figure 3.4 – Phosphorus fractionation measurements from the O, A, and B soil horizons. Soil samples were collected during the summer of 2001 from plot located in the Coweeta Hydrologic Laboratory, Otto, N.C. The plots were located within cove hardwood areas.

Resin = resin extractable P; bic Pi = bicarbonate extractable inorganic P; bic po = bicarbonate extractable organic P; naoh pi = Sodium hydroxide extractable inorganic P; naoh po = sodium hydroxide extractable organic P; mhcl = 1M HCl extractable inorganic P; hcl pi = concentrated HCl extractable inorganic P; hcl po = concentrated HCl extractable organic P; residual = residual P remaining after extractions; pt = total P; po = organic P.


Soil Horizon
O
A
B
0
50
100
150
200
250
300
350
mg P/kg soil
Po
Pt
Residual
HCl Po
HCl Pi
1M HCl
NaOH Po
NaOH Pi
Bic Po
BiC Pi
resin

DISCUSSION:

We hypothesized for the distribution of the three mycorrhizal types that ERM fungi would be predominately distributed where the organic N and P fractions were greatest, that ECM fungi would occur within areas of moderate organic N and organic P concentration, and that AM fungi would occur in areas of primarily inorganic N and P. Generally speaking, the data corroborate our hypotheses. For example, ERM fungi were most abundant in areas of higher organic nitrogen, which probably relates to their ability to produce a diversity of exoenzymes enabling them to utilize organic nitrogen sources. These enzymes include glycosidases (Colpaert and Van Laere, 1996; Cairney and Burke, 1998), phosphoric monoester and diester hydrolases (Mitchell and Read, 1981; Leake and Miles, 1996), peptidase (Bending and Read, 1996) and polyphenol oxidase (Bending and Read, 1996). These prior studies demonstrated that the activities of the enzymes produced by ERM were higher than those produced by ECM (Cairney and Burke, 1998) except for polyphenol oxidase (Bending and Read, 1996). It has been shown that ERM are capable of utilizing organic N directly, by the absorption of free amino acids (Bajwa et al., 1985). Therefore, ERM not only are capable of degrading organic compounds into free amino acids, they can utilize these amino acids directly, bypassing the nitrogen mineralization process. These capabilities might play a role in enabling the ERM fungi to inhabit the upper, organic soil layers, as demonstrated in this study.

Rhododendron maximum has a major influence on the soils, like those at our sites, where it occurs (Beckage et al., 2000). Both the leaves (Yang and Wang, 1978) and roots (Simons, 1988) of rhododendron are rich in phenolics and other organic compounds. The release of these substances into the soil increases leaching (Messenger, 1975), soil acidification (Vance et al., 1986), and sequestration of available N in humic compounds (Silvapalen, 1982). Therefore, the

Table 3.4 – Correlations of variables (loadings) with principal components produced by the PRINCOMP procedure over inorganic and organic nitrogen and phosphorus fractions.

	Principal Component 1	Principal Component 2	Principal Component 3
Organic N	0.44	0.62	-0.32
Inorganic N	0.61	0.21	-0.08
Organic P	0.55	-0.28	0.73
Inorganic P	-0.36	0.7	0.6

Figure 3.5 - Principal Component Analysis of mycorrhizal fungal DNA recovery and soil N and P characteristics. a) Plot of principal components 1 and 2. b) Plot of principal components 1 and 3. c) Plot of principal components 2 and 3. Each "e" represents a recovery of ectomycorrhizal fungal DNA from a soil sample. Each "r" represents a recovery of ericoid mycorrhizal fungal DNA, and each "a" represents a recovery of arbuscular mycorrhizal fungal DNA from a soil sample. PC1 represents organic N and P and inorganic N. PC2 represents organic N and inorganic P, and PC3 represents organic and inorganic P.

Figure 3.5a

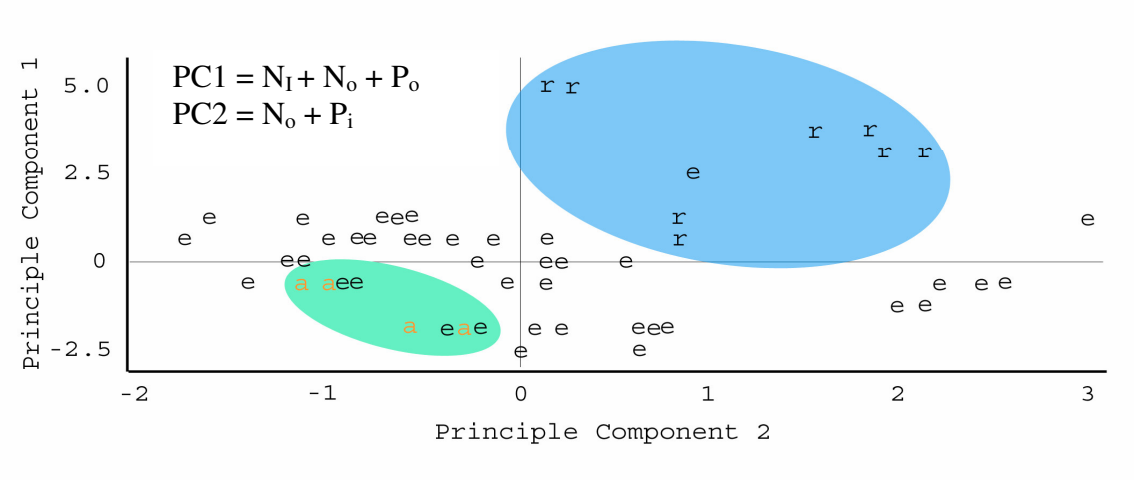


Figure 3.5b

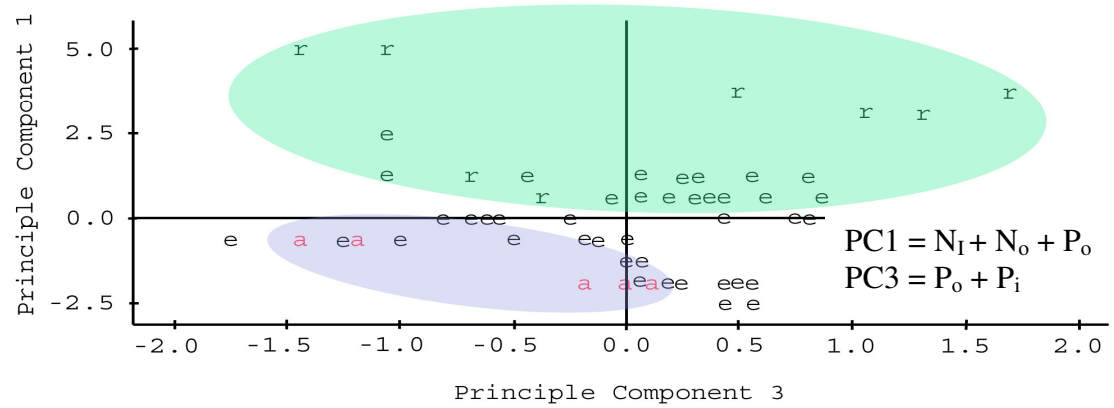
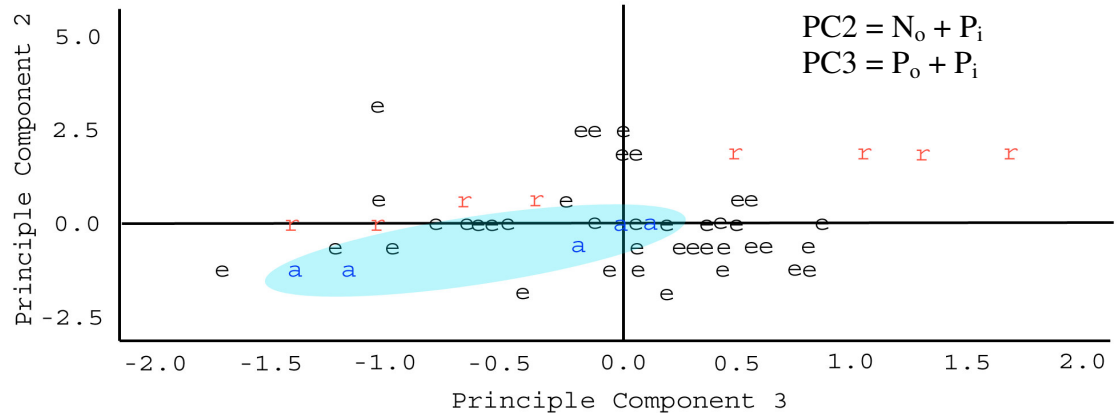


Figure 3.5c



upper soils layers that developed under the *Rhododendron* canopy at our study site would consist of highly recalcitrant litter, acidic humic material, and high levels of polyphenols, all of which ERM are capable of utilizing as a nutrient source. Beckage et al. (2000) found that the litter layer biomass under *Rhododendron* thickets near our study site, were 20% greater than at locations just outside of the thickets. ERM fungal abundance also corresponded to soil areas of greater inorganic nitrogen (Figure 5). ERM fungi have a high affinity for ammonium and nitrate uptake (Read, 1996), and can grow equally well on either mineral N form. With enhanced ability to utilize organic substrates as well as nitrate and ammonium N, ERM fungi would be best adapted to the litter and humic soil layers under a rhododendron canopy. In our study, the vertical distribution of ERM fungi was in the upper soil layers, principally the O horizon, where the organic residues of the rhododendron litter would be the most concentrated.

Arbuscular mycorrhizal fungi at our study site were negatively correlated with organic phosphorus (Figure 5). This isn't surprising because AM fungi only have minimal capacity for the utilization of organic substrates, with the ability to produce phopshomonoesterase (Joner and Johansen, 2000). Therefore, AM fungi would not be able to compete with other mycorrhizal types or with saprotrophic fungi in soil layers consisting of higher organic P levels. There was no obvious pattern of AM fungal vertical distribution with respect to N. AM fungi can utilize both nitrate and ammonium (Hodge, 2001), and the absence of a relationship between AM fungal distribution and soil N in our study may be more indicative of the importance that P plays in AM function than of anything else.

ECM fungi occurred throughout the soil layers, but were most abundant within the A horizon, an observation which may relate to the ability of ECM to access organically bound nutrients. ECM fungi possess a broad array of exo-enzyme production ability. Abuzinadah and Read (1986) analyzed several species of mycorrhizal fungi for their ability to utilize various

organic nitrogen sources. The variability in the ECM capacity to use different organic N substrates led Abuzinadah and Read (1986) to label some ECM as "protein-fungi" and others as "non-protein fungi". The "protein-fungi" decomposed and utilized a broad array of organic substrates, while the "non-protein fungi" demonstrated an almost complete lack of any ability to use organic N sources.

Generally, ECM fungal degradative exo-enzyme capabilities are less than those of ERM fungi (Cairney and Burke, 1998), with the possible exception of polyphenol oxidase (Bending and Read, 1996). Because Bending and Read (1996) measured increased polyphenol oxidase activity on just a single species wider generalizations cannot be made, but a proportion of the ECM fungal community possessing this higher polyphenol oxidase activity than ERM would help explain the vertical soil distribution of ECM fungi that we observed in this study. Because the *Rhododendron* litter is very high in phenolics (Yang and Wang, 1978; Simons, 1988), ECM fungi more proficient at utilizing phenolics as nutrient sources could utilize this specialized niche and explain how ECM could compete with ERM in the upper soil horizon. ECM fungi were also located in the B soil horizon competing with AM for the inorganic nutrients. The “non-protein” fungi of Abuzinadah and Read (1986) lacked a capacity to utilize organic substrates as nutrient sources and were dependent upon inorganic nutrients. The ECM recovered from the B horizon could correspond to this ECM type.

Given the spatial distribution of ECM fungi between organic and inorganic layers, we expected to be able to distinguish which fungal species occurred in each soil layer. However, there were no clear trends correlating a specific fungal species to a nutrient environment. This could be explained in several ways. First, our assignment of sequences to a mycorrhizal species or genus is only an approximation, as there were sequence differences between our samples and the Genbank database. We assigned sequences to their closest Genbank match based upon the

phylogenetic trees, and since the Genbank database contains a relatively small proportion of known fungal sequences, misrepresentations based upon the Genbank database are possible. Lastly, it is possible that the sequences we recovered and assigned to mycorrhizal identifications were recovered from dormant hyphae, spores, or mycorrhizal fungi that were existing in a saprotrophic state. It is possible that various mycorrhizal fungi can persist in a non-symbiotic state during portions of their lifespan (Read and Perez-Moreno, 2003) and function in a way which differs from to their symbiotic state.

Since the ERM fungi occur in the upper soil layers, and have access to the greatest concentration of all N and P forms, and the AM fungi are located in the lower soil horizons in this mixed-mycorrhizal type forest we studied, it is reasonable to believe that this may be evidence of niche differentiation (Dickie et al., (2003). The ERM fungi are the most capable of acquiring nutrients from organic substrates, and were most abundant in areas where the greatest concentration of these nutrients occur. AM, lacking the same capacity to acquire organic N and P are subjugated to the lower fertility B horizon. Ectomycorrhizas, however, occur in all soil horizons, and throughout the range of N and P distribution. This may be due to the extreme diversity of ECM fungi. With many thousands of species thought to exist (Molina et al., 1992), it seems likely that this diversity in numbers would be matched by a functional diversity in nutrient acquisition and competitive ability. This agrees with Goodman and Trofymow (1998), in that they found that ECM existed in all substrate types, from organic to inorganic. Specifically, they recognized that *Cenococcum geophilum* was found more often in the O horizon than mineral soil layers. *Piloderma* species were found in areas where decayed wood was more prevalent. Other ECM types, *Rhizopogon vinicolor* and *Lactarius rubrilacteus*, were equally abundant in the organic and mineral soil horizons. We found no clear differences in the distribution of ECM with respect to N and P.

Not surprisingly, the interaction of environmental factors and mycorrhizal distribution in the forest we studied is more complicated than our hypotheses predicted. N and P fraction distribution didn't fully explain the mycorrhizal type distribution in our soil. There are a number of other factors known to effect mycorrhizal distribution, including soil moisture (Anderson et al., 1986), pH (Coughlan et al., 2000) soil structure and temperature (Kernaghan and Harper, 2001), O_2 and CO_2 levels (Bruns, 1995). These factors require further study for their role in determining mycorrhizal distribution in soils, and cannot be ruled out here.

Increased sample size and improvements in our molecular protocol would assist in further understanding the spatial distribution of mycorrhizal fungi, and the factors effecting their distribution. Given the low sample size accompanying the data of ERM and AM mycorrhizal types as compared to ECM, improvements are required in primer and amplification efficiency. Once a better sampling of the mycorrhizal population structure is obtained, the distribution of the ERM and AM types might be altered from those presented here. We had significant problems with the ITS1F/ITS4 primers acquiring ERM sequences and with the AM primer sets amplifying AM sequences.

SUMMARY:

To our knowledge, this is the first attempt to determine the vertical soil distribution of all three dominant mycorrhizal types (AM, ERM, and ECM fungi), and to determine the factors which create this distribution. We analyzed whether mycorrhizal fungal distribution was correlated with N and P fraction distribution. Our results indicate that there is much variation in the vertical distribution of ECM mycorrhiza which is not correlated to these N and P fractions. ECM fungi occurred throughout the range of N and P fraction distributions, and it is likely that there are differences in preferred soil environment even within ECM groups. The AM fungi and

ERM fungi had more confined distributions. AM fungi occurred predominately within the B soil horizons corresponding to lower organic P and N concentrations. This spatial distribution in the soil profile corresponds to the documented ability of AM fungi to utilize organic N and P sources as nutrient substrates. ERM occurred in the upper, organic soil horizons, likely due to its high diversity and activity of exo-enzymes and the capacity to directly uptake amino acids. However, there were methodological concerns that require addressing before future studies utilize similar protocols to evaluate mycorrhizal soil distribution. AM and ERM DNA suffered from small sample sizes due to poor primer performance and limited PCR amplification. However, the data did demonstrate that soil N and P fraction distribution is associated with mycorrhizal distribution. Additional research incorporating protocol improvements at finer scales of N and P assessment are needed to further evaluate the dynamics of the mycorrhizal community.

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CHAPTER 4

TOPOLOGICAL MEASUREMENTS OF ROOTS COLONIZED BY ERICOID, ECTO- AND ARBUSCULAR MYCORRHIZAL FUNGI[1]

[1] Rachel, G.C. and R.L. Hendrick. To be submitted to *New Phytologist*

ABSTRACT

Root topology and mycorrhizas both play a key role in plant nutrient acquisition. How topology and mycorrhizal colonization interact may be key in understanding the symbiosis. We investigated the impact that different mycorrhizal types, ericoid (ERM), ecto- (ECM), and arbuscular mycorrhizal (AM) fungi have on root system architecture in a soil at Coweeta Hydrologic Lab, Otto, N.C. The soil studied maintained host plants for all three mycorrhizal fungi, *Rhododendron maximum* (ERM), *Tsuga canadensis* (ECM), *Liriodendron tulipifera* (AM). Using PCR,sequencing, and BLAST searches, we identified the different mycorrhiza occurring on roots of each of the three host species. We then used scanned images of each root fragment to determine several topological parameters including altitude, magnitude, and total exterior path-length. We then compared the topology of roots colonized by different mycorrhizae. Our results indicate that different ECM groups do not have a noticeable effect on the parameters measured. Other comparisons could not be made due to a lack of AM and ERM diversity on the roots tested. AM Tulip poplar roots produced the most herringbone topology with a log Pe/log magnitude ratio of 1.5. ERM Rhododendron roots had the most dichotomously branched topology with a log Pe/log magnitude ration of 1.47, while ECM hemlock produced roots with an average ratio of 1.48.

Introduction

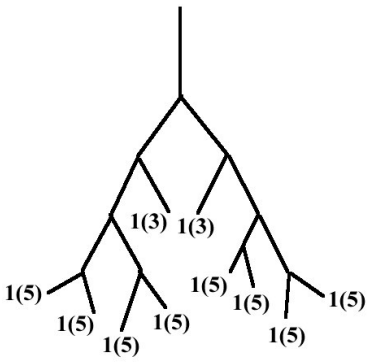
Root Architecture is comprised of three components: topology, size and position (Lynch, 1995). Topology is defined as the configuration of the segments of the root system with respect to each other (Fitter and Strickland, 1991). For plant root systems these components are usually considered to be the segments of roots between branch points. The segments are called links and the branch points are called nodes (Fitter, 1985). The topology or configuration of these components is defined using a variety of measurements (Fitter, 1991), including link length, lateral branch angle, radial branch angle, link magnitude, pathlength, and altitude. Three numbers, magnitude, pathlength, and the altitude can characterize a specific, unique topology. Magnitude is the number of links that has developed from the link of interest; pathlength is the number of links from the exterior link of interest to the resource sink defined for the root systems under study; altitude is the number of links in the longest individual pathlength from resource sink to the most outer link. Given these three variables, a single functionally significant topology can be recorded indicating the fundamental branching pattern of a root system.

Theoretically, there are two extremes to root topology (Fitter, 1985), "herringbone" and "dichotomous" (Figure 4.1). The herringbone type consists of a main taproot with unbranched lateral roots. The herringbone type is less transport efficient than the dichotomous due to the fact that nutrients transported from the laterals must all pass through the main axis (Fitter,1985). Additionally, the links arising from the main axis at the root tip must pass materials through a high number of links to reach the sink, or the top of the root system (Fitter, 1985). However, this topology increases the volume of soil explored by the root system and minimizes overlap of depletion zones between laterals (Fitter, 1985). Thus, the herringbone topology is typical of root systems located in low nutrients soils, where resource acquisition is typically diffusion limited (Fitter, 1991).

Figure 4.1 - Examples of the “herringbone” and “dichotomous” topologies. Each root has a magnitude of 10. For the same magnitude, the herringbone topology has greater total exterior path-length and altitude than the dichotomous topology. Numbers not in parentheses represent a single root tip. Number within parentheses represent the pathlength from the point of origin to the root tip.

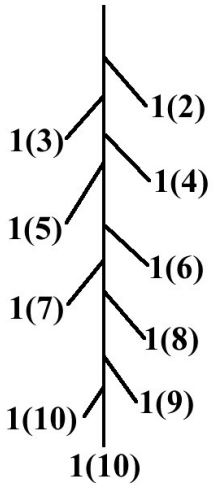
Dichotomous

Altitude = 5
Total Pathlength = 46
Magnitude = 10



Herringbone

Altitude = 10
Total Pathlength = 64
Magnitude = 10



The dichotomous system is a highly branched rooting structure with many lateral roots of multiple orders (Fitter, 1985). This system, with its smaller diameter roots and highly branched structure, is more transport efficient, as each link passes its nutrients through a minimum number of links to reach the sink (Fitter, 1985). The dichotomous structure has more exterior links (absorbing links) at a lower total root magnitude; this means the dichotomous topology has more absorbing capacity, and transport capacity per unit of root biomass (Fitter, 1991). Due to this topology's increased absorbing capacity, it is typical of soil areas with abundant nutrients, where competition for resources requires root systems to access available nutrients (Fitter, 1985).

The impact of ectomycorrhizae (ECM) on root topology is well known (Gerdemann, 1971). ECM causes root elongation and root hair formation to be suppressed due to the formation of the ectomycorrhizal mantle. The short lateral roots typical of ECM hosts undergo dichotomous branching, forming several orders of laterals and corolloid structures. This increase in branching seems to result from hormonal interaction between the fungus and root (Kaska et al., 1999). Thus, the expected result of ECM colonization is an increase in root branching and a more dichotomous topology better adapted at absorption of materials from the fungal hyphae. However, few studies have utilized topological analysis to investigate the architectural characteristics of ECM roots.

Arbuscular mycorrhizal (AM) fungi seem to modify plant root topology in various ways. Farley and Fitter (1999) found no change in root topology in herbaceous perennials after AM colonization, while Hodge et al., (2000) demonstrated that roots of *Plantago lanceolata* increased production in organic patches upon addition of AM innoculum. Cui and Caldwell (1996) reported decreased growth of *Agropyron desertorum* roots upon addition of arbuscular mycorrhizae, while Torrisi et al., (1999) describes an increase in root density of cotton roots following mycorrhizal colonization. There are conflicting topologies recorded for tree species as

well. Pregitzer et al., (2002) found that AM *Liriodendron tulipifera* roots were thick and unbranched with a low specific root length, while AM *Acer saccharum* roots were much thinner and extensively branched. Clearly, there is no uniform topological response of AM roots to mycorrhizal infection. Hetrick et al., (1988) found decreased root branching in *Andropogon gerardii* colonization by AM under low phosphorus levels, and suggested that this might result from the root system acquiring an architecture adapted for soil exploration, r and one primarily relying on the mycorrhizal fungus for absorptive tissue. Hetrick et al., (1988) also suggested that under appropriate soil nutrient conditions, the mycorrhizal fungi might directly control plant root branching by hormone production.

The impact of ericoid mycorrhizae (ERM) on root topology is unknown. Ericoid mycorrhizal roots are referred to as "hair roots" due to their extremely small diameter (Read, 1996) and form a dense layer of fibrous roots near the soil surface (Dodd et al., 1984). However, the impact of the production of these hair roots on topological parameters is unknown.

The purpose of this study was to quantify the topological condition of field grown ECM *Tsuga canadensis,* ERM *Rhododendron maximum* and AM *Liriodendron tulipifera* root systems, and to determine if different fungal taxa within each mycorrhizal type affected host topology. All of the measurements were conducted on field extracted root systems; we therefore could not measure a non-mycorrhizal root system. Instead, comparisons were made between root systems of different species and roots of the same species with different fungal communities. My hypothesis was that there would be differences in topology between roots of the same species when the mycorrhizal species assemblages are dissimilar. For example, hemlock roots with a certain mycorrhizal community may be more "dichotomous" than roots associated with a different mycorrhizal community. To test this hypothesis we collected root samples from several forest plots inhabited by hosts of ECM, AM, and ERM fungi. We also collected roots from

different soil horizons to determine if the different mycorrhizal communities at different soil depths, or the soil nutrient status, effected root topology.

METHODOLOGY:

Sampling Sites:

The soil samples utilized in the study were collected at the Coweeta Hydrologic Laboratory located in Otto, North Carolina (35°02'44"N, 83°27'09"W). The sampling plots were located in a cove forest area with Humic Hapludult soil, a *Liriodendron tulipifera* overstory, *Tsuga canadensis* in the sub-dominant canopy to understory position, and *Rhododendron maximum* understory. *L. tulipifera* is associated with arbuscular mycorrhizae (AM), *T. canadensis* forms ectomycorrhizae (ECM), and *R. maxima* forms ericoid mycorrhizae (ERM).

Root Sampling:

Four sampling plots of $25m^2$ in size were located in the cove forest areas. In each plot, four subplots of $900cm^2$ were randomly located. Within each subplot root samples were collected from three soil horizons. The sampling involved digging a soil pit through the B-horizon and collecting representative samples of roots from the soil pit for O, A, and B soil horizons. Root samples were stored on dry ice for transport back to the laboratory.

Root samples were washed free of soil by hand, and root material from each sample was recovered to the extent possible. Roots were then laid flat on a paper sheet and scanned into a computer using a standard flatbed scanner. Five to ten root tip samples from each root fragment were collected and freeze dried for DNA extraction. The entire process from thawing to freeze-drying took approximately 3 hours for each sample,. The scanned images were utilized to determine the root topological parameters, altitude (a), magnitude and total exterior pathlength (Pe) after Fitter (1985). Altitude is the number of links in the longest path from the roots point of

origin to the furthest root tip. Magnitude it the total number of root tips. Total exterior pathlength is the sum of all paths from the root point of origin to each root tip. Each scanned root segment included approximately 150 links. Diameter and link lengths were not measured due to the difficulty involved in measuring these parameters from a scanned image and because of the time constraints in measuring the diameter and lengths of nearly 16000 total links.

DNA Extraction, PCR, and Sequencing:

DNA extraction on the freeze dried root tip samples were carried out using the protocol of Karen (1998). In brief, freeze dried tissue was immersed in a solution of SDS and B-mercaptoethanol, and disrupted using a micropestle. The solution was then purified with phenol-chloroform and cleaned using standard ethanol precipitation procedures. The resulting DNA was utilized in two subsequent reactions. First, the rDNA region was PCR amplified using the ITS1F/ITS4 primer set and protocol of Gardes and Bruns (1994). The amplified material was purified using a Wizard purification kit (Promega 2003) and then sequenced on an ABI 3700 sequencer using BigDye 2.0 chemistry (Applied Biosystems, 2003). The resulting data were compared to the Genbank database using the BLAST software application. E-values, which indicate the probability of a sequence matching a database sequence by chance, accounting for sequence length and database size, were used as the indicator of match quality. E-values less than e^{-100} were selected as matches, and an appropriate taxonomic identification inferred from the matches. E-values of 0 were accepted as exact matches. The second reaction utilized the primer set PRC and PRD (Brunner et al., 2001) to amplify the chloroplast gene from the root tissue to identify the plant species. The reaction conditions follow the protocol of Brunner et al. (2001) and included a 25µl reaction volume with 1.4mM $MgCl_2$, 0.1mM of each nucleotide, 0.25U of *Taq* polymerase (Fisher), and the following thermal profile: 3 minutes at 94ºC, and 40 cycles of

94ºC – 1 min, 57ºC – 2 min, 72ºC – 2 min, with a final extention of 10 min at 72ºC. The reaction products were purified and sequenced and analyzed in the same manner as the ITS fragments.

The data were then grouped by plant species, soil horizon, and associated mycorrhizal fungi. The differences in the topological parameters were assessed over these categories using the analysis of variance procedure (ANOVA) in SAS 8.2 (SAS, 2003).

RESULTS:

One hundred and fifty root tips were collected for plant and fungal DNA from the sample plots. After the species of each root was determined by PCR, 106 samples belonged to one of the plant species of interest; hemlock, rhododendron, or tulip poplar. From these 106 samples, 8 groups of mycorrhizal fungi were recovered by DNA extraction and sequencing (Table 4.1). The topological parameters of the root segments of each mycorrhizal type were compared to each other using analysis of variance to determine if mycorrhizal species affected root topology.

There were no significant differences between the topological parameters of roots from different soil horizons. Therefore, the data were pooled by plant species and mycorrhizal group. There were, however, topological differences between roots of each plant species tested (Table 4.2). Roots of tulip poplar had the most herringbone topology of three species (Figure 4.2) The ratio of log Pe/ log magnitude indicates the branching extent of a root system while accounting for the root systems size, and a greater log Pe/ Log magnitude number indicates a more herringbone topology. Tulip poplar roots had the highest log Pe/ log magnitude ratios (1.51) of the three species. Tulip poplar roots were generally less branched, having high Pe (2280) and magnitude (192.7) values, with a high average altitude (24.1). Hemlock roots were intermediate in topological scope, with log Pe/log magnitude ratios ranging from 1.46 to 1.51 for the 6 different ECM mycorrhizal groups (Figure 4.2). Hemlock Pe averaged 1717.9, altitude averaged

Table 4.1 - Sequences recovered from root fragments and their possible identity and mycorrhizal type. The "Matching OTU" column indicates either the identity of the closest BLAST match or the soil derived OTU with which the root sequence aligned using Lasergene's Seqman program. E-values for BLAST matches are given. For sequence matches to root OTU's, the percent matching identity level is >90%.

Root sequence	Matching Soil OTU	BLAST or OTU match and accession #		Mycorrhizal Type
1	*Russula virescens*	OTU 11		ECM
2	*Amanita vaginata*	OTU 18		ECM
3	*Endogone sp.*	OTU 2		AM
4	*Hymenoscyphus sp.*	OTU 42		ERM
5	*Tomentella sp.*	OTU 10		ECM
6	Uncultured mycorrhizal fungus	e^{-92}	AY394903	ECM
7	*Cenococcum geophilum*	$5e^{-81}$	AY112935	ECM
8	*Suillus tomentosus*	$9e^{-88}$	AF323117	ECM

Table 4.2 - Topological measurements of roots of each host species. Altitude is the number of links in the longest path from source to sink. Magnitude is the summation of all exterior lengths and Pe is the total exterior pathlength, or the summation of the number of all links. Numbers with the same letter are not significantly different from each other (α=0.05). Standard deviations are in parenthesis.

Host Species	Myc. Type	Mycorrhizal OTU	n	Altitude	Magnitude	P_e
Hemlock	ECM	*Russula sp.*	15	23.6ab (1.8)	147.0ab (49.2)	1478.6ab (177.6)
Hemlock	ECM	*Amanita sp.*	11	24.2ab (3.0)	146.3ab (90.8)	1540.6ab (756.0)
Hemlock	ECM	*Suillus sp.*	12	22.5ab (1.3)	135.3ab (81.2)	1534.9ab (741.6)
Hemlock	ECM	*Cenococcum sp.*	9	23.1ab (3.1)	139.9ab (74.3)	1724.8ab (1693.5)
Hemlock	ECM	*Tomentella sp.*	10	23.8ab (2.8)	141.7ab (75.8)	1596.7ab (1073.5)
Hemlock	ECM	uncultured mycorrhizal fungus	6	22.2ab (3.4)	176.8ab (86.1)	2431.7ab (2086.4)
Tulip Poplar	AM	*Endogone sp.*	18	24.1a (4.0)	192.7a (122.4)	2801.6a (1815.7)
Rhododendron	ERM	*Hymenoscyphus sp.*	21	19.3b (7.9)	124.0b (81.1)	1182.4b (777.5)

23.3, and magnitude had an average of 147.8. *Rhododendron maximum* roots were far more dichotomous than the other two root types; its log Pe/log magnitude ratio was 1.47 (Figure 4.2), its Pe was 1182.4, altitude was 19.3, and magnitude was 124.0. Plots of the Pe /magnitude ratio provide a graphical representation of topological class (Figure 4.3). Data trend-lines that are closer to the solid line (maximum Pe) are more herringbone than those that deviate, and again show that Tulip poplar was more herringbone than the other two species.

Since we only had single mycorrhizal groups for AM and ERM roots, we could not compare the influence of different AM and ERM fungi. We could do so for the ECM, since we had six ECM mycorrhizal groups, but there were no significant differences between mycorrhizal group altitude, magnitude or Pe (Table 4.2). Therefore different groups of ectomycorrhizal fungi apparently didn't cause any topological changes in the hemlock root system.

DISCUSSION:

The root system topology of each plant species we studied was consistent with its mycorrhizal type. The roots of AM tulip poplar had the most herringbone structure (Figure 4.2), which is consistent with the effects of AM colonization (Hetrick et al., 1988). Previous work on tulip poplar root architecture (Pregitzer et al., 2002) at these same forest sites showed that this species possessed thick, unbranched roots with larger diameters than the 8 other forest trees studied (*Acer saccharum, Juniperus monosperma, Picea glauca, Pinus edulis, Pinus elliotii, Pinus resinosa, Populus balsamifera, Quercus alba*). The thick, unbranched herringbone topology would enable the tulip poplar root system to forage the greatest soil volume with a minimal investment in root tissue construction and maintenance (Fitter, 1991). The fungal

Figure 4.2 – Topological Index (log Pe/log magnitude) for the roots of each mycorrhizal group. Group numbers 1,2,5,6,7,8 are ectomycorrhiza hemlock roots. Group number 3 is the arbuscular mycorrhizal tulip poplar group. Group 4 is ericoid mycorrhizal rhododendron group. A higher log Pe/log magnitude ratio indicates a more herringbone root topology because, by definition, a herringbone structure has the greatest number of exterior links in relation to total links.

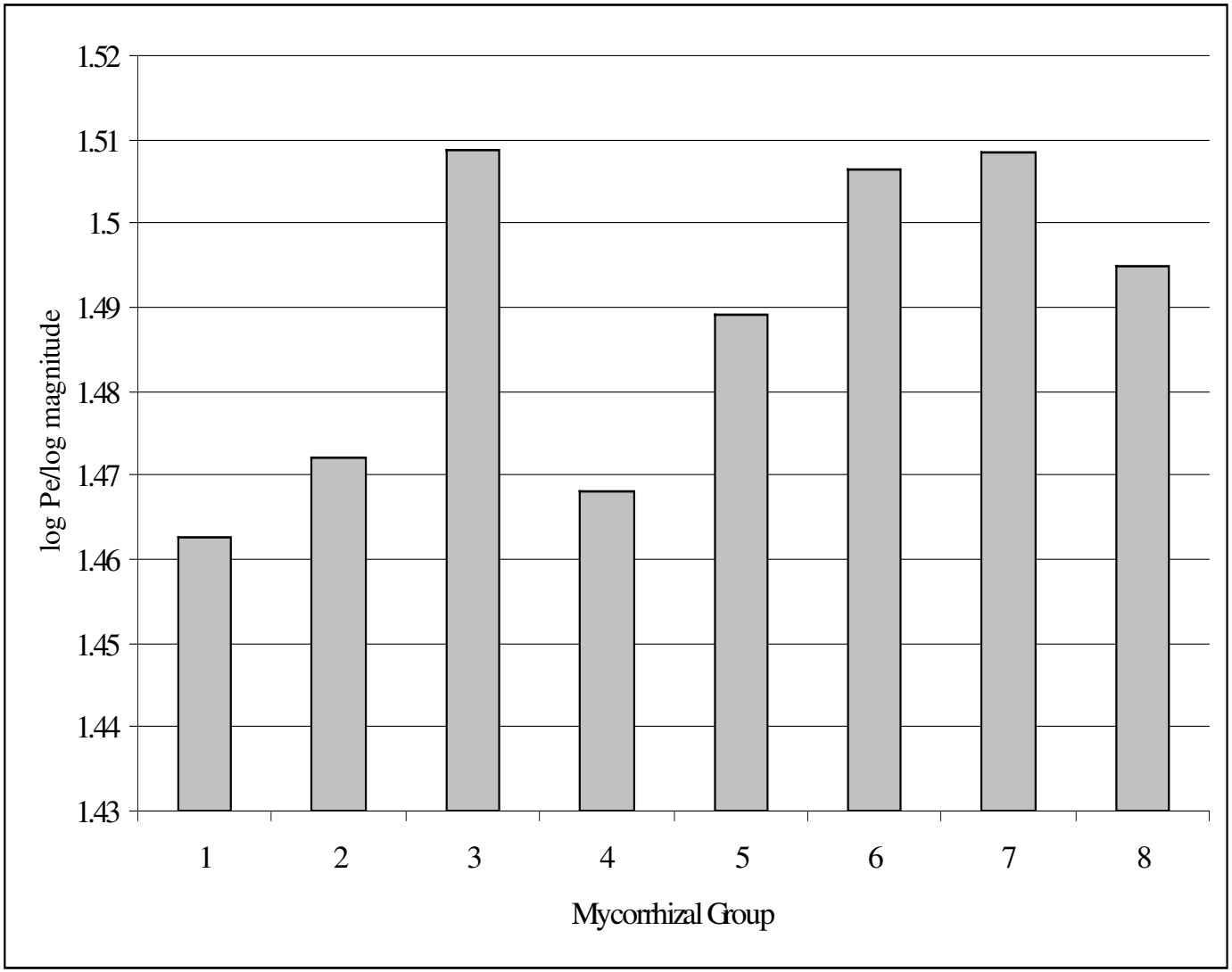
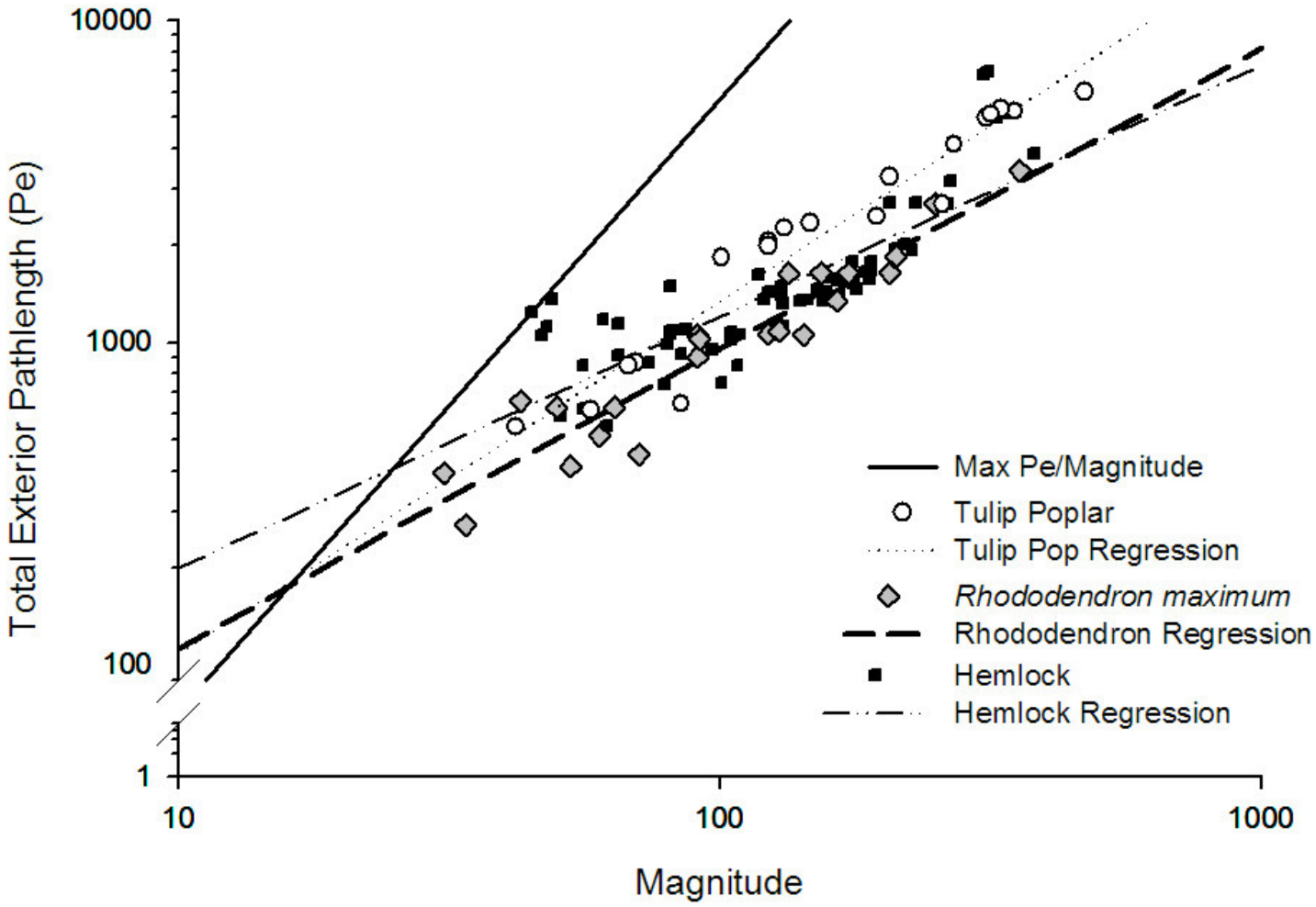

1.52
1.51
1.5
1.49
1.48
1.47
1.46
1.45
1.44
1.43
log Pe/log magnitude
1
2
3
4
5
6
7
8
Mycorrhizal Group

Figure 4.3 – Graph of total exterior pathlength : magnitude ratio for each measured root segment. The plot accounts for variability in size of the root fragments measured. The solid diagonal line represents the maximum possible Pe for a given magnitude. Data points closer to the solid diagonal line are root fragments displaying a more herringbone topology, those further from the line are more dichotomous. The trend-lines associated with plant species indicate how far from "herringbone" the root fragments become as they grow larger in magnitude.


10000
1000
100
1
Total Exterior Pathlength (Pe)
10
100
1000
Magnitude
Max Pe/Magnitude
Tulip Poplar
Tulip Pop Regression
Rhododendron maximum
Rhododendron Regression
Hemlock
Hemlock Regression

hyphae of the mycorrhizae act as the primary nutrient absorbing fine roots of the plant (Hetrick et al., 1988), while the root tissue would provide nutrient transport to and from the plant, as well as placement of the hyphae within the soil (Lynch and Brown, 2001).

Hemlock roots had a more dichotomous topology than tulip poplar roots (Figure 2). The more extensive branching resulted, in part, from the development of bifurcated short roots and corolloid structures typical of ectomycorrhizae (Smith and Read, 1997). While lacking the exploratory ability of a herringbone topology (Fitter, 1991), the dichotomous structure would maximize the root system's transport capability (Fitter, 1985). The ECM fungal hyphae become the exploratory structures of the plant root, with rhizomorph production extending hyphal foraging significant distances into the soil matrix (Agerer, 1995).

ERM *Rhododendron maximum* roots had the most dichotomous topology of the three species studied (Figure 2). An intensely branched root system is typical of Ericaceous plants and their ericoid mycorrhizae (Read, 1996). Ericoid colonization of roots is associated with the production of "hair roots" which form a fibrous, dense root system typically close to the soil surface (Read, 1996). The benefits of this topological type to the plant are the same as those produced by ECM, with the exception that ericoid mycorrhizae don't produce the rhizomorph structures enabling hyphal soil exploration. However, this absence of rhizomorphs is countered by the close proximity of the hair roots to the upper organic soil layers and the greatest concentration of available soil nutrients (Lynch and Brown, 2001).

Despite the occurrence of different ectomycorrhizal fungi on root segments, there were no apparent differences in root topology that were related to fungal taxa. It is possible that different ectomycorrhizal species in general have similar effects on root topology, or that the few species considered here were similar enough in their effect that no topological changes could be detected. It is also possible that the topological influence resulting from colonization of these

ECM resulted from a nutrient mediated impact of ectomycorrhizas on topology, or a plant controlled effect (Hetrick, 1991).

Nutritional effects on root branching and elongation have been well documented. Low nutrient availability increases specific root length (Fitter, 1985), while high N concentrations may increase branching and root length (Drew and Saker, 1978). Zhang and Forde (1998) identified a gene in *Arabidopsis* that initiates lateral proliferation when the root is in the presence of nitrate. Therefore, if each ectomycorrhizal species exploring the same soil volume provided a similar amount of N or other nutrients to the plant root that impact lateral initiation, the topological impacts might be similar.

Plant control of branching during mycorrhizal colonization has been studied by investigating the role of plant-produced hormones (Kaska et al., 1999). Hormone control of ectomycorrhizal root branching has been extensively studied (Gogala, 1991; Barker et al., 1998), but the mechanisms which control the branching, as well as the details of the fungus/plant interactions are still unresolved . Kaska et al. (1999) tested several different chemicals, including auxin and ethylene, for their ability to cause ectomycorrhizal-like root development in uncolonized pines roots. They found that several chemicals could be involved in inducing the dichotomous branching in colonized root tips and that nutrient levels mediated the extent of this branching. Whether the production of these chemicals is controlled by the plant or fungus and their origin is unknown (Smith and Read, 1997).

It is possible that other architectural parameters, which were not measured, did vary with species of ECM fungi. Parameters that were not measured include link length, branching angle, and link diameter (meaning lateral size)(Fitter, 1985). Hetrick et al. (1998) showed that AM colonization could significantly increase root length and diameter. Branching angle is a critical component affecting the exploratory capabilities of the herringbone topology because it impacts

the ability of lateral root tips to grow out of depletion zones formed around the parent root (Fitter, 1985). Branching angle also affects the topsoil foraging ability of some species and as a result, their P efficiency (Lynch and Brown, 2001).

Further topological assessment is needed of the root system of plants supporting all types of mycorrhizal fungi. Root topology may have impacts on nutrient acquisition, plant competition, and growth efficiency (Koide et al., 2000). Given the importance of root topology and architecture, much more information needs to be gathered on this topic. We performed all measurements of topology by hand, which was a labor intensive process and limited the scope of information capable of being gathered from a root system. It is possible that a more intensive measurement process might better quantify the unique topological characteristics of each ECM group. Subsequent studies will include root diameter, link lengths and branching angles as part of the plant root topological assessment. The lack of a non-mycorrhizal control for each plant type was problematical as well. Given the architectural differences likely to exist between mature trees and seedlings, as well as the plethora of other soil variables that might affect root topology, attaining reliable controls for such studies is difficult.

SUMMARY:

We measured the basic topological traits of AM tulip poplar, ECM hemlock, and ERM *Rhododendron maximum* root systems recovered from a forested stand at the Coweeta Hydrologic Lab, Otto North Carolina. Tulip poplar and *Rhododendron* roots were colonized with a single group of AM and ERM, while hemlock roots were associated with six different groups of ECM fungi. The topological measurements of the AM tulip poplar roots matched several previously reported experiments of the topological effects of AM colonization (Hetrick et al, 1998), in that the roots were more herringbone in structure than the ECM or ERM root

systems. *Rhododendron maximum* ERM roots were the most dichotomously branched root systems, which is typical for ERM plants (Read, 1996). ECM roots were slightly less dichotomously branched than ERM roots. The six different species of ECM produced statistically similar topology. The failure of the six ECM species to produce different topological changes in the hemlock root system might be a result of the fungi's similarity or an indication that the control of mycorrhizal root branching is under plant control and of limited variability.

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CHAPTER 5

SUMMARY

Our study site focused on three major tree and shrub species, each of which is capable of forming one of the major mycorrhizal associations. Eastern hemlock (*Tsuga canadensis*), in combination with the proper fungi can form ectomycorrhizas (ECM), tulip poplar (*Liriodendron tulipifera*) can form arbuscular mycorrhizas (AM), and *Rhododendron maximum* can form ericoid mycorrhizas (ERM).

We evaluated mycorrhizal soil distribution and root topology. Both factors can affect host plant nutrient levels, and hence, plant productivity (Smith and Read, 1997; Lynch and Brown, 2001). More specifically, the physical location of mycorrhizal fungal hyphae within the soil can influence hyphal nutrient absorption effectiveness, and hence the quantity of nutrients they have available to transfer to the plant (Read, 1993). Similarly, root topology impacts the position of the root in the soil, and the plant's capacity to forage for soil nutrients (Fitter, 1985). Therefore, the interaction of these two systems is potentially quite important in better understanding forest productivity, but it has been relatively seldom researched. To our knowledge, this is the first attempt to concurrently determine the vertical soil distribution of all three dominant mycorrhizal types (AM, ERM, and ECM fungi) in soils and their correlation with soil N, P, and root topology.

The vertical distribution in soil of the mycorrhizal fungi was determined using DNA-based techniques. The first stage of our study involved assessing five protocols for extracting DNA from soils. This was done to try to eliminate bias introduced by an inefficient extraction technique. Of the five protocols, a commercially available kit (FAST DNA SPIN kit for Soil

QbioGene) was the most effective at providing a large quantity of DNA that was easily amplified with PCR. Using this extraction protocol, we recovered DNA from three soil horizons in sixteen subplots located equidistant to each of the three host species.

After cloning and sequencing of the DNA we identified 18 mycorrhizal fungal species throughout the O,A, and B soil horizons. Twelve of the fungal species were ECM fungi, three were AM fungi, and three were ERM fungi. The ECM fungi occurred predominately within the A soil horizon, although they were found in both the O and B horizons as well. AM fungi were almost exclusively found in the B soil horizon; 83% of the AM DNA was recovered from this horizon. Sixteen percent of the AM fungal DNA recoveries came from A horizon soil. The majority of ERM fungal DNA was recovered from the O horizon (67%), and 33% was recovered from the A soil horizon.

This distribution may be a result of "niche separation" as discussed by Dickie et al. (2003), because each of the three mycorrhizal types has unique enzymatic capabilities to utilize organic substrates. There are thousands of species of ECM fungi (Molina et al., 1992), and this diversity in number is matched by this group's diversity in degradative enzyme production (Read and Perez-Moreno, 2003). The range of enzymes produced by ECM fungi enables them to utilize a wide range of organic substrates as nutrient sources (Abunindah and Read, 1986). In contrast, AM fungi are only known to produce a single class of enzyme aimed at organic phosphorus acquisition (Joner and Johanssen, 2000). This lack of enzyme diversity correlates well with their location in the lower soil horizons, which are typically less concentrated with organic residues (Lynch and Brown, 2001). ERM fungi produce a variety of high activity enzymes such as proteases and lignases that enable them to utilize a diversity of substrates as nutrients sources (Colpaert amd Van Laere, 1996). In general, ERM are superior to ECM in organic substrate utilization, probably explaining their prevalence in the O soil horizon.

In the second part of our study, we measured the concentration of several different organic N and P fractions in the O,A, and B soil horizons. We then used Principal Component Analysis to analyze the relationship between mycorrhizal fungal soil distribution and N and P concentration. Since ECM fungi were wide-ranging throughout the soil horizons, there was no specific correlation of soil distribution with N and P. AM fungi were associated with lower N concentrations, and ERM were correlated with higher N and inorganic P levels. These data seem to corroborate the concept of "niche separation" discussed above. That is, ERM fungi were present in the soil horizon with the highest concentrations of organic N and P, while AM fungi were recovered from the soil horizon with the lowest organic N and P concentrations. ECM fungi were present in all three soil horizons, probably indicative of their ability to produce a variety of degradative enzymes.

The final stage of our study investigated the association between root topology and the mycorrhizal fungal population of the root. Using root fragments from the same soil plots used in part 1 and 2 of this study, we identified the mycorrhizal fungi colonizing the root system, and three of its topological parameters; altitude, magnitude, and total exterior pathlength. Single taxonomic groups of AM and ERM fungi colonized tulip poplar and *Rhododendron* roots, respectively. We, therefore, could not determine if different taxons were correlated with different root topologies. Six different taxonomic groups colonized ECM roots, but analyses of the taxonomic groups and the topological data did not show any topology differences related to different fungal taxa. Instead, the only significant differences in topology were those among plant species. Tulip poplar (AM) was the most "herringbone", or sparsely branched root system, *Rhododendron* (ERM) was the most dichotomously branched root system, and eastern hemlock (*Tsuga canadensis*) was slightly less dichotomous in structure than the *Rhododendron*. The

topological nature of the roots systems studied compares with the architectural characteristics of these species reported previously (Pregitzer et al., 2002; Read, 1996).

One caveat for our fungal hyphal distribution study is that the number of AM and ERM fungi recoveries from the soil samples were much lower than the number of ECM fungi recoveries. This low level of representation of AM and ERM fungi from our soil samples provides uncertainty as to the actual fungal distribution in the soil profile we studied. Further development work on DNA extraction and especially PCR amplification of AM and ERM fungi from soils are needed.

Summary: Our study assessed the vertical distribution of ECM, AM and ERM fungi in a soil profile at Coweeta Hydrologic Laboratory, Otto, NC. We also investigated the topological characteristics of root systems within this soil when colonized by different mycorrhizal taxonomic groups. Our results showed a relationship between mycorrhizal type and soil depth. ERM fungi were recovered from the O soil horizon, ECM occurred in all soil horizons, and AM were predominately recovered from the B horizon. We observed no topological differences between ECM root systems colonized with different taxonomic groups of ECM fungi. Topological changes between the root systems of the three plant species were observed as expected. Our interpretation of these results is that niche differentiation is likely a significant factor in mycorrhizal fungal spatial distribution, with the capacity of the different mycorrhizal fungal types to produce degradative enzymes a key in explaining this distribution. Topological differences weren't observed for root systems colonized by different mycorrhizal fungal taxonomic units. However, more extensive sampling and manipulative experiments are required before any conclusions can be drawn.

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