EXPLORING A PLANT-SOIL-MYCORRHIZA FEEDBACK WITH *RHODODENDRON MAXIMUM* IN A TEMPERATE HARDWOOD FOREST

by

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(Under the Direction of Ronald L. Hendrick)

ABSTRACT

Rhododendron maximum is altering plant diversity and composition in southern Appalachian forests, but the mechanisms by which it does so are not fully understood. *R. maximum* may alter the nitrogen (N) cycle and create a N-based plant-soil-mycorrhiza feedback. Standing stocks of soil organic matter and inputs of leaf and root litter were greater in forest microsites with *R. maximum* than those without. Tannin extracts from *R. maximum* litter had a relatively high capacity to precipitate protein compared to extracts from tree litter. Across the growing season, soil inorganic N availability was generally lower in *R. maximum* soils. Our data suggest that *R. maximum* litter alters N cycling through the formation of recalcitrant protein-tannin complexes. We examined the soil fate of reciprocally-placed ^{15}N enriched protein-tannin complexes. Based upon recovery of ^{15}N from soil N pools and microbial biomass, protein-tannin complexes derived from *R. maximum* leaf litter were more recalcitrant than those from hardwood trees. Ericoid mycorrhizal roots of *R. maximum* were more enriched in ^{15}N compared to ecto-and arbuscular mycorrhizal roots, particularly with *R. maximum* derived protein-tannin complexes. These results suggest that *R. maximum* has greater access to the N complexed by its own litter tannins compared to other forest plants and trees. We characterized the composition of the ericoid

mycorrhizal root fungal community of *R. maximum* using both a culture-based and cloning-based approach (direct DNA extraction and amplification of the ITS region) and observed 71 putative fungal taxa. Fungi include ericoid symbionts *Rhizoscyphus ericae* and *Oidiodendron maius*, several potential symbionts in the Helotiales, Chaetothryiales and Sebacinales, several ectomycorrhizal taxa, saprotrophs and a plant pathogen. This fungal community may aid in the breakdown of complex organic substrates by producing extracellular enzymes such as laccase. We characterized a portion of the laccase gene sequence from genomic DNA of cultured root fungi. Fungal taxa possessed one to four unique laccase gene sequences and sequence polymorphisms were not related to fungal taxonomy. A N-feedback between *R. maximum,* soils and mycorrhizal fungi may contribute to both the expansion of this shrub and the concomitant suppression of other plant species in southern Appalachian forests.

INDEX WORDS: ericoid mycorrhiza, polyphenols, nitrogen, extracellular enzymes, ITS sequence, laccase

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Plant-soil feedbacks influence plant community structure and nutrient cycling in terrestrial ecosystems (Ehrenfeld et al., 2005). Nutrient feedbacks can involve the biotic and abiotic components of ecosystems and resolve themselves on many spatial and temporal scales. Plant litter nutrient feedbacks with Nitrogen (N) are common as litter chemistry directly impacts decomposition and mineralization processes (Chapin, 2003). These plant-soil N feedbacks are extraordinarily complex as they involve N movement through soil food webs (Wardle, 1999) and the acquisition of N by mycorrhizal fungi and plants (Näsholm et al., 1998). Plants and mycorrhizal fungi can directly assimilate both inorganic and organic forms of N, and some mycorrhizal fungi actively breakdown and access N in complex organic substrates (Read et al., 2004). Therefore, plant-soil-mycorrhiza feedbacks seem plausible since plants alter the N cycle by virtue of their litter chemistry, and plants and mycorrhizal symbionts create niches in their acquisition of N sources (McKane et al., 2002).

Ericaceous vegetation impacts forest systems

Ericaceous vegetation inhibits the regeneration of trees in both natural and managed forests world-wide (Mallik, 2003). For example, in northern Sweden, *Empetrum hermaphroditum* Hagerup. inhibits growth of *Pinus sylvestris* L. (Steijlen and Zackrisson, 1987), *Kalmia angustifolia* L. reduces regeneration of *Picea mariana* (Mill.) BSP in eastern Canada (Inderjit and Mallik, 1996), and in western Canada and the Pacific Northwest, USA *Gaultheria shallon* Pursh suppresses growth and regeneration of various conifers (Preston, 1999). In extreme cases

ericaceous plants can shift plant community structure from a forest to a heathland (Mallik, 2003). Ericaceous plants increase in cover due to the suppression of fire (Steijlen and Zackrisson, 1987) and from the creation of habitats after forest harvesting (Mallik, 1993). Reductions in forest regeneration may be due to the presence of allelopathic compounds in ericaceous litter that inhibit seed germination (Pellissier, 1993) and alterations to the soil environment such as increased organic material and decreases in pH and nutrient availability (DeLuca et al., 2002; Castells et al., 2003). Although the influence of ericaceous plants on forest communities are well documented, the precise mechanisms by which they alter patterns in plant community structure are not always clear (Mallik, 2003)

Litter polyphenols

The influence of litter polyphenols on the N cycle is one potential mechanism of tree suppression by ericaceous understories (Hättenschwiler and Vitousek, 2000). Concentrations of litter polyphenols are often negatively correlated with soil fertility or forest productivity (Nicolai, 1988; Northup et al., 1995; Côté, 2000). Ericaceous plants, in particular, contain high concentrations of polyphenols in foliage and roots (Gallet and Lebreton, 1995; Preston, 1999), and tannins, a type of polyphenol, precipitate with forms of organic N creating complexes in senescing plant material and in soils (Kraus et al., 2003b). However, tannin concentrations alone are not indicative of protein precipitation capacity, as the composition and stereochemistry of these molecules also directly influence complex formation (Kraus et al., 2003a).

Protein-tannin (P-T) complexes are recalcitrant (Howard and Howard, 1993) and the accumulation of these complexes in soils can decrease decomposition rates (Basaraba and Starkey, 1966; Benoit et al., 1968), increase organic matter content (Handley, 1961) and lower N mineralization rates (Bradley et al., 2000; Fierer et al., 2001; Castells et al., 2003; Kraus et al.,

2004a). The reduction of N availability may contribute to the suppression of ectomycorrhizal (ECM) or arbuscular mycorrhizal (AM) forest trees that rely upon the availability of soil inorganic N, while ericaceous plants themselves may be less affected as they are hypothesized to access P-T complex-N through their ericoid mycorrhizal (ERM) symbionts. Therefore, ericaceous plants may control N cycling by stimulating the formation of P-T complexes, excluding other plant species from this portion of the N pool and creating a plant-soil-mycorrhiza feedback (Northup et al., 1998; Preston, 1999; Mallik, 2003).

Ericoid mycorrhizal fungi

In support of a plant-soil-mycorrhizal feedback hypothesis, ERM fungi of ericaceous plants can degrade and access P-T complex N to a greater extent than ECM fungi in pure culture studies (Bending and Read, 1996a; Bending and Read, 1996b; Bending and Read, 1997). However, in a pot study, there was no difference among ERM, ECM and AM plants in the acquisition of P-T complex N (Bennett and Prescott, 2004). In fact, no unequivocal evidence of this feedback exists in nature and our interpretation of pure culture studies is limited. Two well documented ERM fungal species, *Rhizoscyphus ericae* W.Y. Zhuang & Korf, (formerly *Hymenoscyphus ericae*) and *Oidiodendron maius* Barron, were assumed as primary associates of ERM roots, and have been the focus of pure culture research. However, ERM fungal assemblages contain a broader diversity of taxa than previously believed, due to the advent of DNA-based techniques that directly identify root fungi (Berch et al., 2002; Perotto et al., 2002; Allen et al., 2003). Therefore, the structure of ERM root fungal communities is only beginning to be understood, and potential for these diverse communities to acquire N from complex organic substrates, such as P-T complexes, remains unknown. Based upon pure culture research, the two known ERM fungi from this community, *R. ericae* and *O. maius*, are highly saprotrophic, and produce extracellular

enzymes at a greater level than ECM and AM fungi (Read et al., 2004). Polyphenol oxidases are a group of oxido-reductase enzymes that degrade recalcitrant organic substrates (Leonowicz et al., 2001; Burke and Cairney, 2002) and polymerize toxic phenols (Bending and Read, 1997). These enzymes may contribute to the release of protein from P-T complexes (Burke and Cairney, 2002), however, there is no information about soil enzyme activities of polyphenol oxidases in forest soils with ERM understories, nor any direct evidence that these enzymes aid in the liberation of the organic N associated with P-T complexes.

Laccase

Laccase is a polyphenol oxidase enzyme produced by many fungi in the Basidiomycota and Ascomycota, including of leaf and wood decomposers and mycorrhizal symbionts (Dix and Webster, 1995; Bending and Read, 1996b; Chen et al., 2003; Valderrama et al., 2003; Luis et al., 2004). The fungal laccase gene spans ca. 1,800 bp, with an open reading frame of 1,554 bp and four highly conserved copper binding domains (Eggert et al., 1998). Fungal laccases appear to have evolved from a single ancestor, however individual fungal species often possess several isozymes (Gonzalez et al., 2003; Lyons et al., 2003; Luis et al., 2004) and these laccase gene nucleotide sequences are polymorphic (Valderrama et al., 2003). In previously studied saprotrophic basidiomycetes, one species can possess as many as five laccase isozymes (e.g. *Trametes* spp., Hoshida et al., 2002). High numbers of laccase isozymes appear to be more common among saprotrophic versus ECM basidiomycetes (Luis et al., 2004). Although isozymes are diverse and nucleotide sequences are highly polymorphic, laccase isozymes in the Basidiomycota are species-specific (Valderrama et al., 2003; Luis et al., 2004), suggesting that gene diversity may be the result of independent divergence events (Valderrama et al., 2003).

Despite the potential ecological importance of laccase production by ERM fungi, laccase gene sequences remain uncharacterized in these fungi.

Rhododendron maximum

Rhododendron maximum L., an evergreen sclerophyllous shrub, is distributed across the eastern USA, but is most prevalent in southern Appalachian forests (Monk et al., 1985; Baker and Van Lear, 1998). In this region, *R. maximum* forms dense spreading thickets that suppress regeneration (Phillips and Murdy, 1985; Nilsen et al., 1999; Beckage et al., 2000; Nilsen et al., 2001), reduce plant species diversity (Baker and Van Lear, 1998), leading to a change in forest community structure (Lambers and Clark, 2003). Unlike the temperate and boreal coniferous forests where ericaceous plants impact regeneration, southern Appalachian forests are dominated by broad-leaf deciduous trees, diverse in plant species (Hardt and Swank, 1997) and relatively N rich (Knoepp and Swank, 1998). *R. maximum* cover has increased over the last century due to the loss of *Castanea dentata* (Marsh.) and lack of natural fire (Phillips and Murdy, 1985; Dobbs and Parker, 2004), and may continue to increase in coverage with the loss of *Tsuga canadensis* (L.) Carr. to the hemlock wooly adelgid (Ellison et al., 2005).

The reduction of light under *R. maximum* thickets contributes to the mortality of tree seedlings, however shade is not entirely responsible for these patterns (Clinton and Vose, 1996; Lambers and Clark, 2003). Allelopathic effects on seed germination and inhibition of seed dispersal do not appear important in these forests (Nilsen et al., 1999; Lei et al., 2002), and soil factors such as moisture, temperature, exchangeable cations and pH are only subtly different or not consistently different between thicket and non-thicket areas, nor among studies (Boettcher and Kalisz, 1990; Clinton and Vose, 1996; Nilsen et al., 2001; Clinton, 2003; Beier et al., 2005). However, *R maximum* clearly alters the soil environment with accumulations of organic material

due to slow decomposition of leaf litter (Boettcher and Kalisz, 1990; Hoover and Crossley, 1995). *R. maximum* has tannin rich foliage (Hunter et al., 2003), similar to other ericaceous species that suppress forests regeneration (Preston, 1999). Therefore, *R. maximum* tannin inputs may lead to the formation of P-T complexes in soils, thereby reducing the availability of inorganic N, and contributing to the suppression of tree seedlings in these forest microsites. Furthermore, *R. maximum* may be less affected by this alteration of the N cycle if it has access to P-T complex N through the saprotrophic activities of ERM fungi.

The primary goal of this research was to test the hypothesis of a plant-soil-mycorrhiza feedback with *R. maximum* in southern Appalachian forests. This feedback hypothesis was approached from three different perspectives at different spatial scales. In the first study, we explored characteristics in N cycling between hardwood forest microsites with and without *R. maximum*. We hypothesized that patterns in *R. maximum* litter quantity and quality would indicate P-T complex formation in *R. maximum* forest microsites and we hypothesized that the availability of inorganic N would be diminished in this microsite. Finally, we hypothesized that the activities of extracellular enzymes contributing to P-T complex degradation would be elevated under *R. maximum* because of the presence of ERM fungal communities and the importance of these substrates as an N source.

The second study focused directly on fate of P-T complex-N into soil pools and mycorrhizal roots. We studied the movement of ^{15}N into soil pools and mycorrhizal roots from synthesized ^{15}N enriched P-T complexes that were derived from the leaf litter of forest microsites with and without *R. maximum*. We hypothesized that P-T complexes derived from *R. maximum* tannins would be more recalcitrant than those derived from hardwood trees, and the degradation of both types would be greater in *R. maximum* soils compared to hardwood soils. We also

hypothesized that ERM roots of *R. maximum* would be more enriched in ^{15}N compared to ECM and AM roots of forest trees and plants, providing evidence of a plant-soil-mycorrhiza feedback.

The third study explored the composition and structure of ERM root fungal communities of *R. maximum*. We utilized both culturing and cloning methods to characterize the fungi associating with ERM roots. We hypothesized that ERM roots would be diverse, both taxonomically and in the number of taxa. Since ERM fungi are considered saprotrophic, we hypothesized that ERM fungal richness would be greater in the O horizon compared to the A horizon. We characterized a portion of the laccase gene sequence in ERM fungi because extracellular enzyme production may be an important means for ERM fungi to degrade and access N from complex organic substrates. We hypothesized that ERM fungi would possess a diversity of laccase gene sequences both within and between fungal taxa. Although the presence of laccase gene sequences is not indicative of gene expression, characterizing these nucleotide sequences is a valuable step towards measuring laccase gene expression *in situ* and determining if laccase production is a mechanism by which ERM fungi acquire recalcitrant organic N.

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CHAPTER 2

RHODODENDRON THICKETS ALTER N CYCLING AND SOIL EXTRACELLULAR ENZYME ACTIVITIES IN SOUTHERN APPALACHIAN HARDWOOD FORESTS[1]

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ABSTRACT

Rhododendron maximum L., a spreading understory shrub, inhibits overstory regeneration and alters forest community structure in southern Appalachian hardwood forests. Using paired plots and reciprocal litter transplants in forests with and without *R. maximum* cover, we examined the influence of *R. maximum* on litter mass and quality, N cycling and soil extracellular enzymes. Standing stocks of soil organic matter, soil N, leaf litter mass and fine root biomass were greater in forests with *R. maximum* than those without. Tannin extracts from *R. maximum* foliage, and leaf litter and fine roots collected under *R. maximum* had a relatively high capacity to precipitate protein compared to extracts from trees. Across the growing season, soil inorganic N availability was generally lower under *R. maximum*, mostly due to reduced NO_3^- availability. Our data suggest that *R. maximum* litter alters N cycling through the formation of recalcitrant polyphenol-organic N complexes. Soil extracellular enzymes indicate the potential processing rates of organic substrates. Between forest types, polyphenol oxidase activity was greatest in *R. maximum* O horizons, regardless of litter type, suggesting that the local microbial community can better degrade and access protein-tannin-complexed-N. Protease activity did not differ between forest types, but was greater on *R. maximum* leaf litter than hardwood leaf litter. The alteration of the N cycle via the formation of polyphenol-organic N complexes may contribute to hardwood seedling suppression, while the enzymatic release of these complexes by ericoid mycorrhizal fungi may increase N acquisition for *R. maximum* and contribute to its expansion in southern Appalachian forests.

INTRODUCTION

Ericaceous plants inhibit forest regeneration in systems throughout the world (Mallik, 2003; Nilsson and Wardle, 2005), however, the precise mechanisms by which they do so are unknown (Hättenschwiler and Vitousek, 2000; Mallik, 2003). Plants in the Ericaceae produce foliage and roots rich in polyphenols (Gallet and Lebreton, 1995; Preston, 1999). Plant polyphenols (e.g. tannins) are structurally and functionally diverse (Waterman and Mole, 1994), and influence litter and soil chemistry, and ecosystem N cycling (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003b). Ericaceous understories often result in lower N mineralization and decomposition rates (DeLuca et al., 2002; Read et al., 2004), and polyphenolic concentrations of plant litter are negatively correlated with soil fertility or productivity (Nicolai, 1988; Northup et al., 1995; Côté, 2000).

The formation of polyphenol-organic N complexes, and their influence on the N cycle, may be one mechanism of tree suppression by ericaceous understories. These complexes decrease protein and plant material decomposition rates (Basaraba and Starkey, 1966; Benoit et al., 1968), resulting in organic matter accumulation (Handley, 1961) and lower N mineralization rates (Bradley et al., 2000; Fierer et al., 2001; Castells et al., 2003; Kraus et al., 2004a). Ericaceous plants may control N cycling in ecosystems with low inorganic N availability by increasing the formation of polyphenol-organic N complexes, excluding other plant species from this portion of the N pool, and hence contributing to their suppression (Northup et al., 1998; Preston, 1999; Mallik, 2003). The ericoid mycorrhizal (ERM) fungi of ericaceous plants can degrade and access complexed organic N to a greater extent than can ectomycorrhizal (ECM) fungi (Bending and Read, 1996a; Bending and Read, 1996b; Bending and Read, 1997). Therefore, while altering patterns of N availability for other plant species, ericaceous plants may

access polyphenol-complexed-N through the saprotrophic capacity of ERM fungi. ERM fungi produce extracellular enzymes which can degrade polyphenol-organic N complexes, but soil activities are not well documented in forests containing ericaceous understories.

Rhododendron maximum L., a dominant ericaceous understory shrub of southern Appalachian forests, accounts for as much as 18- 34 Mg ha^{-1} in aboveground biomass (Baker and Van Lear, 1998). *Rhododendron maximum* forms dense thickets that spread by layering and root sprouts (Monk et al., 1985), and thicket cover has increased since fire suppression and the loss of *Castanea dentata* to the Chestnut blight (Phillips and Murdy, 1985). The success of *R. maximum* interests forest ecologists because it suppresses conifer and hardwood regeneration (Phillips and Murdy, 1985; Nilsen et al., 1999; Beckage et al., 2000; Nilsen et al., 2001), reduces understory species diversity (Baker and Van Lear, 1998), and changes forest community composition (Lambers and Clark, 2003). *Rhododendron maximum* leaf litter has a slow decomposition rate relative to the leaf litter of other forest species (Hoover and Crossley, 1995; Hunter et al., 2003), and *R. maximum* increases soil organic matter (Boettcher and Kalisz, 1990), and decreases inorganic N availability (Nilsen et al,. 2001, Boettcher and Kalisz, 1990). Therefore, *R. maximum* may influence soil N processes through its litter quality, possibly through the precipitation of organic N by polyphenols. However, the role of *R. maximum* litter polyphenols on N cycling, and the activities of soil extracellular enzymes that degrade polyphenol-organic N complexes have not been addressed.

We explored the effect of *R. maximum* thickets on N cycling and soil extracellular enzyme activities in a southern Appalachian hardwood forest. First, we evaluated standing soil organic mass and N, litter mass and inorganic N availability. We hypothesized that O horizon mass and N, leaf litter mass and standing root biomass would be greater, and available inorganic

N would be lower in forests with *R. maximum* thickets compared to those without. Our second objective was to evaluate the reactivity of polyphenols from foliage, leaf litter and fine roots. We expected that polyphenols from *R. maximum* plant tissues would have a greater reactivity relative to those from hardwood species. Our final objectives were to measure activities of extracellular enzymes that degrade polyphenol-organic N complexes (polyphenol oxidases, PPO) and organic N (proteases) in soils and on reciprocally placed leaf litter. Since ERM fungi have a greater capacity to produce PPO and proteases in pure culture, than do ECM fungi, we expected greater activities of these enzymes in forest soils with *R. maximum* thickets compared to those without. We also expected that the placement of experimental leaf litter treatments under *R. maximum* would result in greater enzyme activities than those placed in forests without *R. maximum* because of local differences in microbial communities.

MATERIALS AND METHODS

Site Description

Our study sites are mature southern Appalachian northern hardwood forests located in the Coweeta Hydrologic Laboratory (a Long-Term Ecological Research (LTER) site) and Nantahala National Forest in North Carolina, USA (35º 03'N, 83º 25'W, Swank et al., 1988). We established five blocks of paired 5 m x 5 m plots with N to NW aspects along a 2.5 km span of a high elevation ridge (1430–1460 m). The hardwood forest overstory composition is continuous within plot pairs; one plot of each pair contains a dense understory *R. maximum* thicket, while the other does not (hereafter referred to as Hdwd + Rmax and Hdwd plots, respectively). The overstory consists of *Quercus rubra* L., *Betula lenta* L. and *Betula alleghaniensis* Britt., *Acer rubrum* L., and *Fraxinus americana* L. The Hdwd plot understory is dominated by *Acer pennsylvanicum* L., *Amelanchier arborea* (Michaux f.) Fernald, and *Castanea dentata* (Marshall)

Borkh. Annual herbs and ferns are present, but the Hdwd plots lack ERM host plants. In contrast, Hdwd + Rmax plots contain a dense *R. maximum* thicket and are devoid of other understory species and herbs, with the exception of occasional achlorophylous plants, *Monotropa uniflora* L. and *Conopholis americana* L. Wallroth. Soils are Inceptisols, formed in residuum of igneous and metamorphic rock, including the Burton, Plott, Craggey series (Humic and Humic Lithic Dystrudepts), and soil series are consistent within each block. Depth to lithic contact ranges from 20-80 cm. Two blocks are adjacent to the LTER watershed 5-27 which has a mean annual temperature of 9.4 ºC, and evenly-distributed annual precipitation of over 250 cm (L. Swift, unpubl. data).

Organic horizon stores and soil nutrients

In each plot, three randomly located samples of organic horizons and fine woody debris (FWD) were extracted using a square template (0.0613 m^2) and a serrated knife. Samples were separated into three categories; FWD (bark, twigs and stems < 10 cm diameter), Oi horizon, and a pooled Oe and Oa horizon (Oe/a), and dried at 60ºC. Soil samples (5 cores per plot, homogenized by horizon) were dried, ball-mill ground and analyzed for total C and N (Micro Dumas combustion analysis). We measured pH on a subset of unground Oe/a and A soils (H_2O; 1:10 O horizon, 1:5 A horizon). The A horizon soils were further measured through atomic absorption spectrophotometry for exchangeable cations (K, Mg and Ca; NH_4OAc extraction) and available P (double acid extraction) (Robertson et al., 1999).

Inorganic N availability

We assayed N availability using a resin bag approach (Binkley and Matson, 1983). Resin bags contained 2.5 g wet mass of both cation and anion exchange resin beads (AG 50W-X8 H^+ and AG 1-X8 Cl^-, Bio-Rad, Hercules, CA) sewn into square nylon bags (2.5 x 2.5 cm), re-charged

with 1N HCl and rinsed with DI water. For each monthly measurement, four resin bags were randomly placed in each plot of four blocks, ~ 5 cm below surface into a 45º angled slice, targeting a high density root zone while minimizing the severing of roots above the bag. Bags were removed after 28 days, rinsed with DI water, and individually extracted with 1M KCl. Extracts were analyzed for NH_4^+-N and NO_3^- -N, and corrected with blank bags, using a continuous flow colorimetric assay (Technicon AutoAnalyzer). Resin-bag extractable N (NH_4^+ and NO_3^-) was measured once in 2004 (September) and monthly during the 2005 growing season (April - August).

Leaf litter inputs and root biomass

We collected current year leaf litter in October 2004, when leaf fall was complete, from three randomly-located areas in each plot using a 0.158 m^2 template. Although evergreen, leaf loss from *R. maximum* peaks in the fall (Monk et al., 1985), and we collected only fresh leaf litter (green or yellow) to avoid overestimating *R. maximum* litter inputs. Leaf litter was sorted by species, and dried at 60º C.

We sampled total standing root biomass and peak season root standing biomass (≤ 1 mm), a proxy for root litter inputs (Joslin and Henderson, 1987; Gill and Jackson, 2000) in July 2004. Four randomly collected soil cores (4 cm diameter, 11 cm deep, from each plot), were placed on ice and frozen until sorted. We sorted roots into three size classes (coarse >1 mm, fine 0.5- 1 mm, and very fine < 0.5 mm). Coarse and fine roots were washed, dried at 60 ºC and weighed. Very fine root length was determined using a line-intersect technique, from which mass was determined using specific root length (SRL, cm g^{-1}) (Hendrick and Pregitzer, 1993).

Foliar and fine root total C & N

We sampled green foliage of all commonly occurring tree species by pooling samples from 3-5 individuals of each species at each block in July, 2004. Fine and very fine roots were collected from soil cores (above), sorted from organic material, and pooled by plot. Green foliage and fine roots were washed, kept on ice, flash-frozen in liquid N and lyophilized. All samples were ball-mill ground into a fine powder for total C and N analysis (Micro Dumas combustion) and for tannin extraction (see below).

Tannin protein precipitation

We used the radial diffusion assay to measure tannin reactivity in green foliage, fine roots and leaf litter (Hagerman, 1987). This method provides a relative measure of tannin protein precipitation on a dry mass basis. Foliar and root sampling are as described above. Leaf litter was collected after leaf fall (October 2004) and pooled within each plot, air dried and ball-mill ground into a fine powder. Agarose-protein plates were created as described in Hagerman (1987). We used two protein types, bovine serum albumin (BSA, fraction V, lyophylized powder, Sigma A4503) and gelatin (Knox brand), added to the agarose solution at concentrations of 1 mg (gelatin) or 0.5 mg (BSA) ml^{-1} buffer, respectively. The volume of precipitate from applied 50% aqueous methanol extracts of plant tissues was measured with digital calipers.

Soil extracellular enzyme activities

Polyphenol oxidase (PPO) activities were assayed with 3,4 Dihydroxy-L-phenylalanine (L-DOPA) (Sigma D928) which serves as a general substrate for the oxygen oxido-reductase enzymes laccase (EC 1.10.3.2), catechol oxidase (EC 1.10.3.1) and tyrosinase (EC 1.14.18.1). All enzymes share some degree of overlap in substrate affinity, and oxidize polyphenolic compounds coupled by the four electron reduction of O_2 to H_2O (Thurston, 1994). Protease (EC

3.4.) activity was measured with azocoll (Azo dye impregnated collagen, Sigma A4341) a general protease substrate (Colpaert and van Leare, 1996) that tests for the activity of both exo- and endopeptidases.

From June-August, five soil samples (2 cm diameter) were randomly sampled and homogenized by horizon from each plot. Oi horizon samples were cut into ~1 cm^2 pieces, and Oe/a and A horizons were sieved (4 mm and 2 mm sieve, respectively). Soils were placed on ice, transported to the laboratory and analyzed within 6 h. Extracellular enzymes were extracted with 50 mM NaOAc buffer, pH 5.0 following methods of Decker et al. (1999). A 2 ml aliquot of extract was combined with 2 ml substrate in buffer (10mM L-DOPA or 0.01 g azocoll), for each sample with four analytical replicates, a substrate blank and a soil-free blank. PPO samples were incubated at room temperature in the dark for 1 h, centrifuged and measured at 460 nm. Protease samples were incubated at 37 ºC for 2 h at 200 rpm, placed in ice water, centrifuged and measured at 520 nm. An extinction coefficient for PPO was generated with laccase (Sigma 40442), and protease activity was calibrated against a standard curve using protease (Sigma P6110). Soil subsamples were dried and weighed to correct for water content.

Reciprocal litter extracellular enzyme activity

We conducted a reciprocal leaf litter study to test for the effect of plot (Hdwd + Rmax vs. Hdwd) and leaf litter composition on extracellular enzyme activities. Leaf litter was collected after leaf fall (October 2003), and mixed at four blocks into four leaf litter treatments: 1) *R. maximum* leaf litter only, 2) *R. maximum* and hardwood species leaf litter, 3) hardwood species leaf litter from Hdwd + Rmax plots, and 4) hardwood species leaf litter from Hdwd plots, designated as R, R + H, H (Hdwd + Rmax) and H (Hdwd). At each plot, leaf litter from each of the four treatments were placed into one 15 cm x 10 cm litter bag with 2 mm screen (allowing

for in-growth of roots and fungal hyphae and movement of micro- and mesofauna) and placed randomly at the Oa horizon surface in each plot. We placed wire mesh cages over the bags to prevent incident leaf litter coming in contact with the bag. Extracellular enzymes were extracted from leaf litter after 10 months (August 2004) and 21 months (July 2005), and assayed for PPO and protease activities. Enzyme extraction and analysis were the same as above, except data were expressed on a specific leaf area (SLA, g cm^{-2}) basis to account for the effect of differential decomposition rates on surface area to leaf mass relationships among litter treatments. We used Scion Image software (Scion Corporation, National Institute of Health) to create relationships between mass and surface area.

Statistical analyses

We tested all data for normality. Percent C and N were arcsine-root transformed, and resin bag N data were log transformed. All data were analyzed with SAS software (SAS Institute Inc., Cary NC).

First, using a single-factor (fixed effect) ANOVA with a blocking factor (random effect) design, we tested the effect of plot type (Hdwd + Rmax vs. Hdwd) (n=2) on the following: A horizon nutrient concentration, total leaf litter mass, hardwood leaf litter, FWD, very fine root length, root biomass by root class, root litter, and protein precipitation by leaf litter and fine root extracts. Using the same design, we tested for the effect of plant species (n=8) on protein precipitation by foliar extracts. Tukey's HSD was used as a post-hoc test for significant differences ($\alpha<0.05$) among species.

Second, we used a split-plot (fixed effect) ANOVA with a blocking factor (random effect) design to test for plot and within-plot effects. In this design, the whole-plot factor was plot type (Hdwd + Rmax, Hdwd) and the split-plot factor was either soil horizon (n=2-3), species

(*n*=9), or leaf litter treatment (*n*=4). We examined percent C, percent N, C:N, pH, O horizon mass, and enzyme activities by soil horizon, leaf litter mass by plant species, and extracellular enzyme activities on treatments of reciprocally-placed leaf litter. If within-plot effects were significant, we used a *post-hoc* least square means separation technique.

Finally, we used a two-factor split-plot (fixed effects) ANOVA with a blocking factor (random effect) design to test for the effect of plot type, sampling date and N type (NH_4^+ or NO_3^-) (and the interaction of these factors) on N availability. In this design, the whole-plot factor was plot type (n=2), and sampling date (n=5) and N type (n=2) were the split-plot factors. We used all data from April-August 2005 and due to non-continuous sampling we excluded data from September 2004. If interactions were present, data from each sampling date were analyzed separately to test for the effect of plot and N type.

RESULTS

Organic horizon stores and soil nutrients

The storage and chemistry of O horizons was altered in the presence of *Rhododendron maximum* thickets. The mass of the O horizons was significantly greater in the Hdwd + Rmax plots (P<0.0001) (Table 2.1). FWD in Hdwd + Rmax plots (283.5 (75.9) g m^{-2}, mean (SE)) was not different from that in Hdwd plots (226.9 (44.8) g m^{-2}) (P= 0.51). Both C and N percentages had a significant horizon by plot interaction (both P=0.01), and C:N ratios were significantly different by horizon (P<0.0001) and presence of *R. maximum* (P=0.01), with greater C:N in the O and A horizons in Hdwd + Rmax plots (Table 2.1). Soil pH was not different between plots in the Oe/a horizons, but A horizon pH was lower in Hdwd + Rmax plots compared to the Hdwd plots (P=0.014). A horizon available P (1.01 (0.11) and 0.75 (0.07) mg kg^{-1}), exchangeable Ca (41.4 (15.2) and 61.5 (25.4) mg kg^{-1}), exchangeable Mg (23.6 (5.4) and 21.1 (2.3) mg kg^{-1}), and

exchangeable K (62.1 (7.4) and 54.9 (5.8) mg kg^{-1}) in Hdwd + Rmax and Hdwd plots, respectively, were not significantly different.

Leaf litter and root biomass

Total leaf litter mass was greater in Hdwd + Rmax than Hdwd plots (P<0.0001, Table 2.2). The *R. maximum* leaf litter in Hdwd + Rmax plots was responsible for the difference as total hardwood litter inputs were not significantly different between plot types (P=0.69). *Quercus rubra* and *R. maximum* were the greatest contributors of leaf litter and *Q. rubra* leaf litter mass was not different between Hdwd + Rmax and Hdwd plots (Table 2.2). Total standing root biomass was greater (but not significantly different) in Hdwd + Rmax plots (P=0.26). There were no differences between plots in coarse (P=0.97) and fine (P=0.57) standing root biomass. However, very fine root (<0.5 mm diameter) standing biomass was greater in Hdwd + Rmax plots (P=0.0001) (Table 2.3). The difference in very fine root biomass between Hdwd + Rmax and Hdwd plots, as well as the respective difference in SRL (6957.6 (800.8) and 4188.3 (276.4) cm g^{-1}) resulted in a significant difference in very fine root length density (13,711.2 (775) and 5244.8 (457) m m^{-2}, P<0.0001). Using peak season standing fine root biomass (≤ 1 mm diameter) we estimated greater root litter inputs in Hdwd + Rmax plots compared to Hdwd plots (P=0.0038) (Table 2.3).

Foliar and root C:N

Foliar C:N varied among species (P<0.0001). *Rhododendron maximum* foliage had a significantly greater C:N ratio (45.3 (1.4)) than other species (range 24.7 – 33.0), which were not significantly different from one another (data not shown). Fine root C:N did not significantly differ between Hdwd + Rmax and Hdwd plots (38.9 (2.6) and 35.0 (2.0) respectively (P=0.27)).

Protein precipitation by tannin extracts

Protein precipitation by tannin extracts varied among species for both gelatin and BSA protein (both $P<0.01$) (Figure 2.1). With gelatin, *A. rubrum* extracts precipitated the greatest volume, while *A. arborea*, *B. alleghaniensis*, *B. lenta* and *F. americana* precipitated the least (Figure 2.1). With BSA, *A. rubrum*, *C. dentata*, *Q. rubra* and *R. maximum* extracts precipitated the greatest volume, while *A. arborea* precipitated the least. Hdwd + Rmax plot leaf litter extracts precipitated a greater volume of gelatin and BSA than extracts from Hdwd plot leaf litter ($P=0.01$, $P=0.007$ respectively) (Figure 2.1). Fine root extracts from Hdwd + Rmax plots precipitated more gelatin and BSA than those from Hdwd plots ($P=0.02$ and $P=0.03$ respectively) (Figure 2.1).

Inorganic N availability

NO_3^--N availability peaked in August in Hdwd plots, and peaked in spring in the Hdwd + Rmax plots. In general, NH_4^+-N increased between April and July, and decreased in August. There were significant time by plot ($P=0.046$) and time by N type ($P=0.0008$) interactions with resin bag-captured-N (April – August) (Figure 2.2). Because of the interaction with time, we analyzed each month independently for the effect of plot on NH_4^+-N, NO_3^--N and total inorganic N. In the Hdwd plots, NO_3^--N availability was greater than in Hdwd + Rmax plots in May, June and July ($P<0.05$), while there were only trends of greater NH_4^+-N availability in September ($P=0.13$), April ($P=0.06$), July ($P=0.13$), Aug ($P=0.09$). Total inorganic N was significantly greater in Hdwd plots in April ($P=0.04$) and August ($P=0.01$) and higher, but not significant in both May ($P=0.08$) and July ($P=0.07$) (Figure 2.2).

Soil extracellular enzyme activity

In both plots, PPO activity decreased with soil depth. There was a significant horizon by plot interaction with soil PPO activity (P=0.0005) with greater PPO activity in Hdwd + Rmax plots in the Oi and Oe/a horizons. There was no difference in PPO activity between plots in the A horizon (Figure 3). Soil protease activity decreased with depth (P=0.0042) but was not different between plots (Figure 2.3).

Extracellular enzyme activity of reciprocally-placed leaf litter

PPO activity was greater when litter treatments were placed in Hdwd + Rmax plots compared to Hdwd plots after 10 months (nonsignificant, P=0.13) with a block effect (P<0.0001) (Figure 2.4), and this pattern strengthened after 21 months (marginally significant, P=0.06). Although the effect of litter was not statistically significant (P=0.09), there was a trend of greater PPO activity on the R and R + H litter types compared to the H only litter, especially in the Hdwd + Rmax plots. Protease activity had a strong response to the litter types (P=0.005 and P<0.0001, 10 and 21 months respectively). After 21 months, R litter had the greatest protease activity, followed by R+H and the H litter groups (Figure 2.4).

DISCUSSION

Rhododendron maximum inhibits overstory regeneration

R. maximum clearly inhibits overstory regeneration in southern Appalachian forests, although the contributing factors are not fully understood (Nilsen et al., 1999; Beckage et al., 2000; Nilsen et al., 2001). While reduced photosynthetically active radiation under *R. maximum* contributes to seedling mortality, low light and seedling shade intolerance do not entirely explain the lack of regeneration (Clinton and Vose, 1996; Lambers and Clark, 2003). Additional mechanisms contributing to suppression are not immediately obvious. Inhibition of seed rain and germination

(allelopathy) does not occur (Nilsen et al., 1999; Lei et al., 2002), and soil factors such as moisture, temperature, exchangeable cations and pH are only subtly different or not consistently different between thicket and non-thicket areas, nor among studies (Boettcher and Kalisz, 1990; Clinton and Vose, 1996; Nilsen et al., 2001; Clinton, 2003; Beier et al., 2005). Our results suggest that *R. maximum* influences the N cycle through increases in leaf and root litter, and litter tannins forming complexes with organic N. Furthermore, these changes to N dynamics may not be as detrimental to *R. maximum* if the saprotrophic capacity of ERM fungi is superior to that of the ECM and arbuscular mycorrhizal fungi of hardwood forest trees.

Soil C and N, Litter inputs

Soils under *R. maximum* thickets have greater organic mass and N in the O horizon, and lower percent N in the A horizon, compared to forests without thickets. Hardwood forest O horizon mass in our study is similar to a previous report from a Coweeta northern hardwood stand (615.3 g m^{-2}; Knoepp et al., 2000). *R. maximum* O horizon mass is 280% greater than hardwood forests; similarly, O horizon total N is 286% greater due to both greater O horizon mass and Oe/a horizon percent N. In comparison, in Kentucky, *R. maximum* increased O horizon mass by 9 and 76 %, and O horizon N by 9 and 83% in *Tsuga canadensis* and *Liriodendron tulipifera* cove forests, respectively (Boettcher and Kalisz, 1990). *Rhododendron maximum* thickets vary in density (Baker and Van Lear, 1998), and our selection of dense thickets along with differences in forest community structure may explain the greater impact of *R. maximum* on O horizon mass and N content in our study. In contrast to the O horizons, A horizon percent N is lower in forest soils with *R. maximum* compared to those without.

Organic matter accumulates under *R. maximum* due to greater leaf and root litter inputs and slower decomposition relative to other species. *R. maximum* typically retains leaves for 6

years, losing only 9% of standing crop of leaves annually (Monk et al., 1985), but it increases total leaf litter mass by 43%, and estimated root litter mass by 39% in our study sites. The accumulation of organic matter under *R. maximum* is also due to the slow rate of litter decomposition (Hoover and Crossley, 1995; Hunter et al., 2003). *Rhododendron maximum* foliage is sclerophyllous, and in our study, C:N of *R. maximum* foliage is greater than all other measured species. In previous studies at Coweeta, the DOC:DON ratio of fresh *R. maximum* litter leachate was almost twice that of the dominant tree species (Qualls et al., 1991), and freshly fallen *R. maximum* leaf litter C:N was greater than *Liriodendron tulipifera* and *Quercus prinus* (Hunter et al., 2003). The high C:N in *R. maximum* leaf litter is likely related to low foliar N concentrations, high N remobilization during leaf senescence (Monk et al., 1985), and high concentrations of polyphenols (Hunter et al., 2003). Similarly we found tannin extracts from *R. maximum* leaf litter and roots have a greater protein precipitation capacity than those from hardwood tissues (see below).

Protein precipitation by tannin extracts

Regardless of species, tannin extracts from green foliage precipitated the greatest volume of protein, followed by extracts from fresh roots and leaf litter. From the few existing examples in the literature, tannin concentrations and tannin precipitation capacity are generally greater in foliage than roots of woody plants (Preston, 1999; Kraus et al., 2004b; but see Gallet and Lebreton, 1995) and are even lower in litter and humus (Baldwin and Schulze, 1984; Preston, 1999), possibly due to leaching losses (Baldwin and Schulze, 1984; Nicolai, 1988). *R. maximum* and *Q. rubra* contributed the most leaf litter mass in our study, and both had relatively high foliar precipitation capacities compared to other species. However, *A. rubrum* foliar extracts had the greatest (gelatin), or were among the greatest (BSA) in protein precipitation capacities of species

tested. *Acer* species foliar extracts have high astringency, possibly due to the unique chemistry of their hydrolysable tannins (Bate-Smith, 1977), and *Acer* species foliar tannins have greater reactivity or concentration relative to the northern hardwood species *Q. rubra, F. americana* and *B. alleghaniensis* (Baldwin and Schulz,1984; Côté, 2000; Lovett et al., 2004).

Leaf litter tannin extracts from Hdwd+Rmax plots precipitated more protein than those from Hdwd plots. Relative to Hdwd + Rmax plots, Hdwd plots receive greater leaf litter mass from species whose foliar samples have relatively low protein precipitation (e.g. *Betula* species and *F. americana*), and phenolics from deciduous leaves are rapidly lost during decay relative to evergreen leaves (Kuiters and Sarink, 1987). In Hdwd + Rmax plots, *R. maximum* accounts for over 30% of leaf litter mass and the sclerophyllous nature of *R. maximum* leaf litter allows for greater retention of tannins relative to hardwood leaf litter. In fact, after one year of decomposition, *R. maximum* leaf litter contains greater concentrations of total phenolics, condensed tannins and hydrolysable tannins than *L. tulipifera* and *Q. prinus* leaf litter (Hunter et al., 2003).

Root litter tannins are a significant source of tannins in forest soils (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003b; Kraus et al., 2004a). Even if tannin concentrations in roots are lower than those in leaves, rapid root turnover could make fine roots a greater contributor of soil tannin (Kraus et al., 2003b). In our study, root extracts from Hdwd + Rmax plots precipitate more protein than those from Hdwd plots; therefore, the presence of *R. maximum* roots may contribute to the greater protein precipitation capacity.

Inorganic N availability

We predicted lower availability of inorganic N in forests with *R. maximum* than without because litter polyphenols can decrease the availability of organic N for microbial acquisition and

subsequent mineralization. Resin bag extractable NO^{-}_{3}-N was lower in Hdwd + Rmax plots compared to Hdwd plots during three months of the growing season, while NH_4^+-N and total inorganic N were generally lower overall. Nitrification rates are inhibited by polyphenols (Baldwin et al., 1983) and are negatively correlated with forest stand age, ericaceous cover and soil polyphenol concentrations in coniferous Swedish forests (DeLuca et al., 2002). In the southern Appalachians, the presence of *R. maximum* lowered soil NO_3^- concentrations (Nilsen et al., 2001) and reduced N mineralization rates by 60-100% in cove forests (Boettcher and Kalisz, 1990; but see Nilsen et al., 2001).

Across the Coweeta basin, N mineralization is influenced more by forest type than by climate; forests with *R. maximum* as a dominant species have one of the lowest rates of N mineralization relative to total soil N of those reported (Knoepp and Swank, 1998; Knoepp et al., 2000). Our results support the premise that forest composition can influence inorganic N availability. In forests with *R. maximum* we observed lower inorganic N availability and shifts in soil N distribution across horizons compared to forests without *R. maximum*. Greater N storage in the O horizons under *R. maximum* was related to both greater organic mass and greater percent N in the Oe/a horizons. Since *R. maximum* litter extracts have a greater capacity to complex protein, organic N may be complexed with tannins, increasing stored organic N in the Oe/a horizons and decreasing N mineralization rates. O horizon storage of N under *R. maximum* could also be enhanced by biological immobilization.

Although protein-precipitation capacity of leaf litter tannins can be a strong predictor of N mineralization rates (Handayanto et al., 1997), precipitation capacity does not reflect the recalcitrance of the protein-tannin complex. These complexes differ widely in recalcitrance, possibly due to their molecular structure and composition of condensed and hydrolysable tannins

(Howard and Howard, 1993, Kraus et al. 2003a). The varying strength of polyphenol-organic N complexes may account for the lack of correlation between foliar chemistry, or tannin protein precipitation, and soil N factors in forest ecosystems. Individual tree species influence N cycling through litter quality (Boerner and Kozlowsky, 1989; Boettcher and Kalisz, 1990; Lovett and Mitchell, 2004), yet standard measures of litter quality (e.g. tannin concentrations) are poor predictors of N cycling characteristics because they do not reflect differences in tannin reactivity (e.g. protein-tannin interactions) (Lovett et al., 2004), nor the strength of the protein-tannin complex.

Extracellular enzyme activity

Soil microorganisms degrade organic matter through the production of diverse extracellular enzymes (Caldwell, 2005). Soil extracellular enzyme activities indicate the potential processing rates of organic substrates, allowing for comparisons between forest communities (Allison and Vitousek, 2004). In forest soils, mycorrhizal fungi comprise a significant amount of microbial biomass (Högberg and Högberg, 2002), and among mycorrhizal fungi, ERM fungi may produce more extracellular enzymes, such as PPO (Read et al., 2004). The production of extracellular PPO is limited to some species of leaf litter and bark decomposing fungi, wood rotting fungi, ERM fungi, and some ECM fungi (Dix and Webster, 1995; Read et al., 2004).

We predicted that PPO activity would be greater in *R. maximum* soils because of the presence of ERM fungi and the influence of polyphenols on N availability. In the O horizon, PPO activity was greater in Hdwd + Rmax plots than Hdwd plots. PPO activity is typically greater in litter than soil (Deforest et al., 2004; Gallo et al., 2004), and is positively related to low N mineralization rates, and high litter C:N ratios (Gallo et al., 2004). In a field study, soil PPO activities and concentrations of phenolics were greater under the ECM, polyphenol-rich,

Mediterranean shrub *Cistus albidus* L., compared to areas between plants (Castells and Peñuelas, 2003). Greater PPO activity under *C. albidus* in the above study and in *R. maximum* soils in our study could be attributed to the local microbial community or to greater availability of polyphenolic substrates.

We measured enzyme activities in reciprocally placed leaf litter to test the effects of the local microbial community and leaf litter composition. Although not statistically significant, on average, PPO activity was 21% and 35% greater across the leaf litter treatments after 10 and 21 months, respectively, when they were placed in Hdwd + Rmax plots. Given the high spatial heterogeneity of soil extracellular enzyme activities, it is possible these differences are real and mediated by the local microbial community. The specificity of microbial communities to leaf litter affects the production of extracellular enzymes (Sugai and Schimel, 1993; Sinsabaugh et al., 2002), decomposition and N cycling (Lovett et al., 2004). In hardwood forests of our study, soil PPO may be an important mechanism for polyphenol degradation and nutrient acquisition. ERM fungi of *R. maximum* may contribute to enzymatic degradation processes, although their precise contribution is unknown.

We hypothesized that soil protease activity would be greatest under *R. maximum*; however, protease activity did not differ between forest types. Unlike PPO, most soil fungi produce proteases (North, 1982; Dix and Webster, 1995), including ERM and ECM fungi (Read et al., 2004). On reciprocally placed leaf litter, protease activities displayed a consistent response to leaf litter type with greater activity in *R. maximum* leaf litter compared to hardwood species leaf litter. Protease activity is positively related to leaf litter protein concentration (Lähdesmäki and Piispanen, 1988) and extracellular proteases are induced by the presence of protein in ERM fungi (Leake and Read, 1991). In our study, trotein concentrations may be greater in R litter

compared to H litter after 10 and 21 months of decomposition, inducing the observed patterns in protease activity. These results suggest that protease activity responds to small-scale changes in leaf litter quality, which may have been undetected in the plot level soil assays.

Conclusions

Litter polyphenols may alter the N cycle in *Rhododendron maximum* soils by: forming recalcitrant complexes with organic N, increasing soil organic N content, and lowering inorganic N availability. Ericaceous understories influence N cycling in several ecosystems world-wide; however, most are cold temperate or boreal, and considered N-limited (Nilsson and Wardle, 2005). Our results suggest that an ericaceous understory alters N cycling in a temperate hardwood forest with relatively high inorganic N availability. The influence of *R. maximum* on N cycling, along with its reduction of photosynthetically active radiation, may contribute to the suppression of hardwood seedling regeneration in southern Appalachian forests. Furthermore, the slow growth and low N demands of *R. maximum*, and the potential for ERM fungi to acquire of polyphenol-complexed-N via extracellular enzymes may explain the shrub's continued success. However, the impacts of polyphenol-organic N complexes on N cycling are not fully understood. Protein-tannin complexes vary in recalcitrance (Howard and Howard, 1993), and we need to understand the turnover and fate of tannin-complexed-N derived from ericaceous plants to clarify its importance in N cycling and plant nutrition in forest ecosystems.

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Table 2.1 Soil characteristics in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax).[a]

	Soil horizon and plot type					
	Oi		Oe/a		A	
	Hdwd + Rmax	Hdwd	Hdwd + Rmax	Hdwd	Hdwd + Rmax	Hdwd
mass (g m^{-2})	546.3 (23.9) a	322.5 (48.9) b	2476 (305.0) a	469.7 (79.6) b		
percent total C	47.9 (0.2) a	43.2 (0.8) b	29.1 (4.1) a	15.3 (1.1) b	8.4 (0.9) a	9.3 (0.6) a
percent total N	1.9 (0.1) a	2.0 (0.08) a	1.2 (0.1) a	0.8 (0.04) b	0.36 (0.04) b	0.46 (0.02) a
C:N	25.3 (1.1) a	21.8 (0.6) b	24.5 (0.8) a	18.8 (0.8) b	23.5 (0.8) a	20.1 (0.8) b
pH			4.08 (0.1) a	4.46 (0.1) a	4.99 (0.2) b	5.68 (0.2) a

[a]Values are means and (SE) from $n = 4$ sampling blocks; different letters between Hdwd + Rmax and Hdwd plots within a soil horizon indicate a significant difference, $\alpha < 0.05$.

Table 2.2. Leaf litter mass and species composition in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax).[a,b,c]

	Leaf litter mass (g m^{-2}) Plot type	
Species	Hdwd + Rmax	Hdwd
Amelanchier arborea	13.0 (3.6) a	10.0 (3.2) a
Acer pennsylvanicum	0	7.0 (1.5)
Acer rubrum	12.2 (3.6) a	20.2 (10.1) a
Betula alleghaniensis	2.3 (1.1) b	10.8 (3.8) a
Betula lenta	22.6 (10.8) b	31.1 (12.3) a
Castanea dentata	0.11(0.1) b	1.5 (0.5) a
Fraxinus americana	0.34 (0.2) b	3.56 (1.9) a
Quercus rubra	146.6 (13.9) a	124.7 (13.0) a
Rhododendron maximum	112.4 (8.6)	0
Total leaf litter mass[d]	336.8 (12.9) A	235.5 (18.6) B

[a]Values are mean and (SE) of $n = 4$ sampling blocks.
[b]Data possessed a significant plot by species interaction ($P < 0.0001$).
[c]Species' leaf-litter mass in the same row followed by a different letter are significantly different between forest plots ($\alpha < 0.05$) and total leaf litter mass by plot is significantly different ($\alpha < 0.05$).
[d]Total leaf litter also contains unidentifiable leaves, and species not included in individual species analysis..

Table 2.3. Standing root biomass and estimate of fine root litter inputs in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax).[a]

	Plot type Root biomass (g m^{-2})	
Root class[b]	Hdwd + Rmax	Hdwd
Very fine	197.1 (11.1) a	125.2 (10.9) b
Fine	98.4 (12.5) a	90.2 (8.0) a
Coarse	203.4 (43.1) a	201.6 (38.9) a
Total	498.9 (47.7) a	417.0 (47.9) a
Root litter[c]	295.5 (19.8) a	215.4 (14.4) b

[a] Values are means (SE), values in the same row followed by a different letter are significantly different ($\alpha < 0.05$); $n = 4$ sampling blocks.
[b] Very fine = <0.5 mm, fine = 0.5-1 mm, coarse = > 1 mm, root litter = <1mm.
[c] Root litter inputs estimated by peak season fine root biomass (Joslin and Henderson, 1987; Gill and Jackson, 2000).

Figure 2.1. Protein precipitation of foliage (by species), and root and leaf litter (by plot) extracts from hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) for a) gelatin and b) BSA protein types. Bars indicated by a different letter (within tissue group) are significantly different ($\alpha < 0.05$) with $n = 5$ sampling blocks.

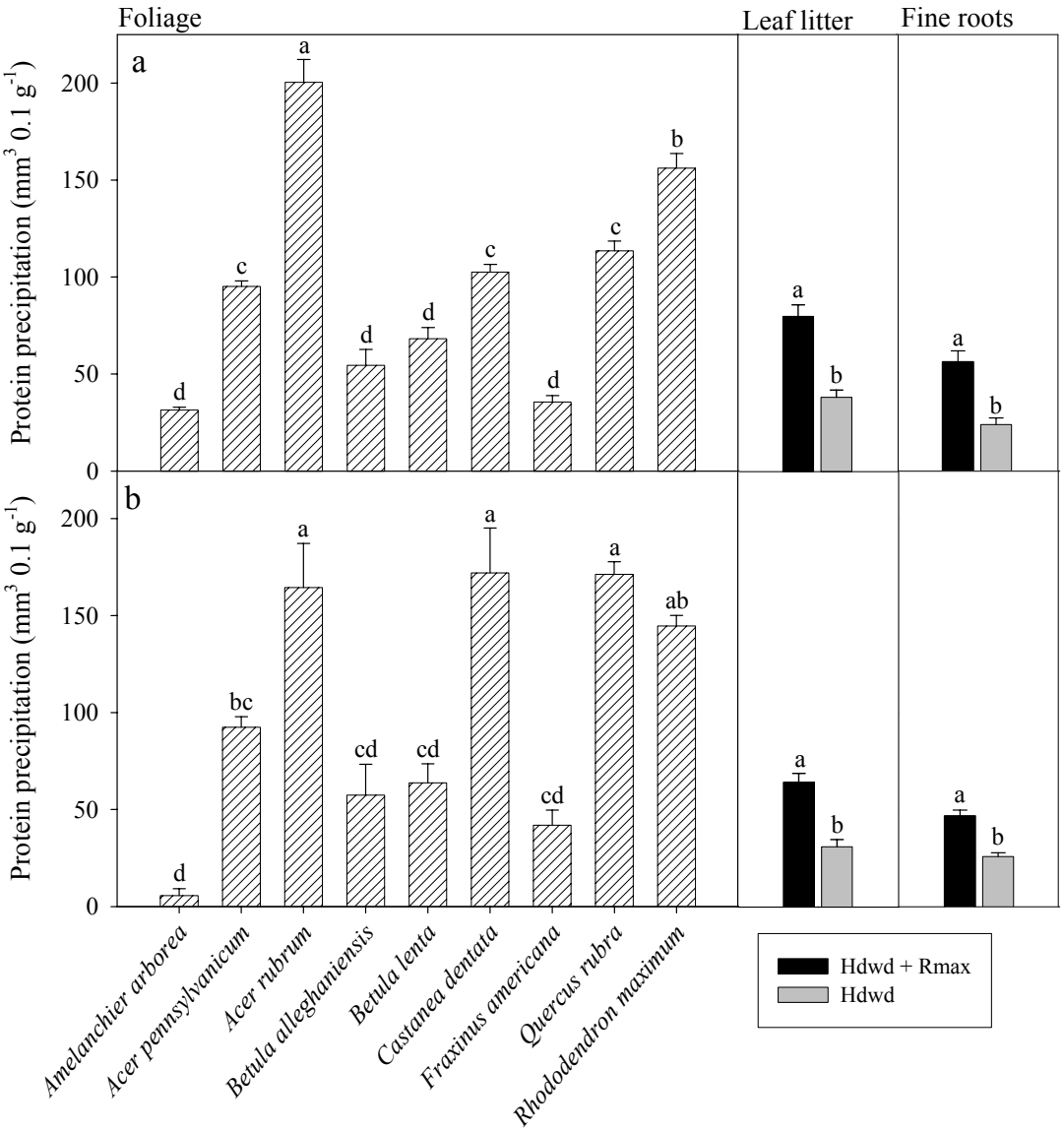


Figure 2.2. Monthly resin bag extractable N in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) for a) NO^-_3-N and b) NH_4^+-N. Asterisks denote a significant difference between plot type at each sampling date ($\alpha < 0.05$); $n = 4$ sampling blocks.

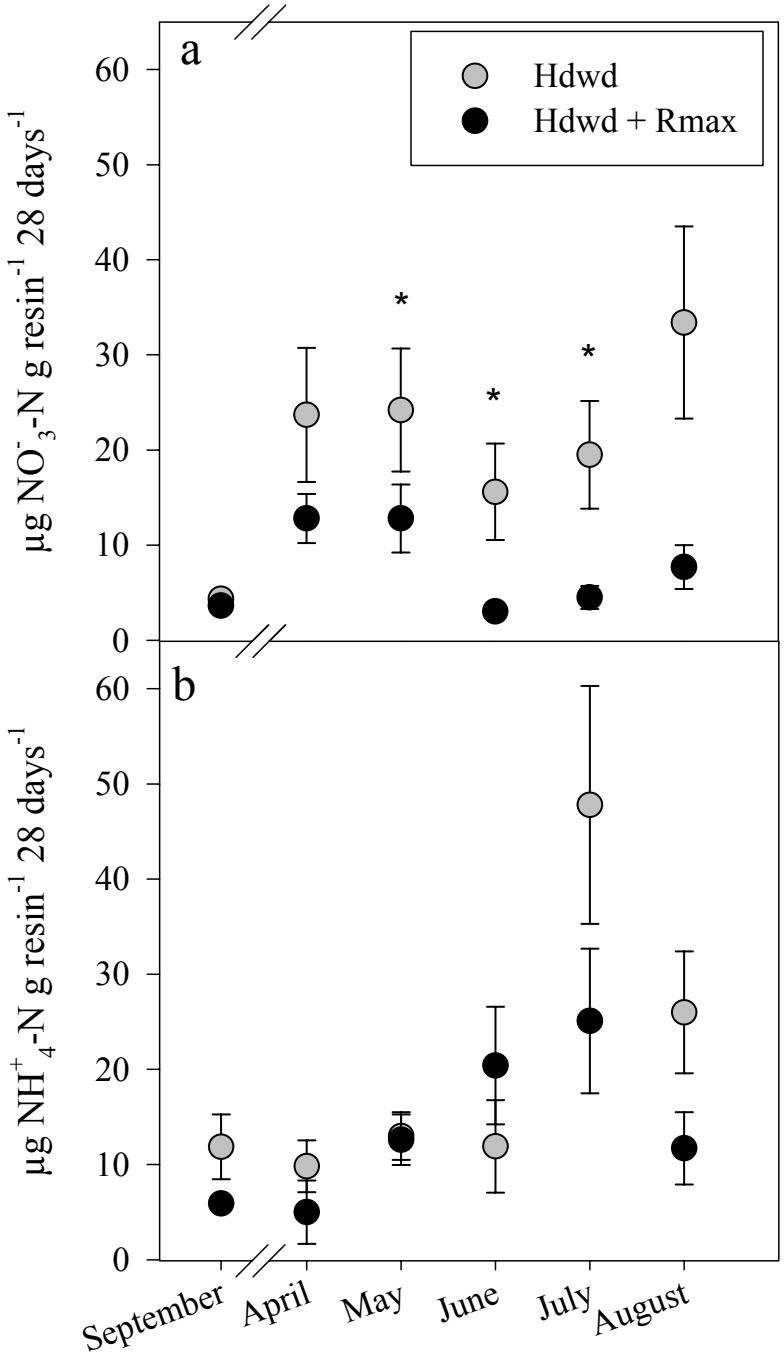


Figure 2.3. Soil extracellular enzyme activities in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) of a) polyphenol oxidase (PPO) and b) protease. Asterisks denote a significant difference in enzyme activity between plot types at each soil horizon ($\alpha < 0.05$*);* $n = 4$ sampling blocks.

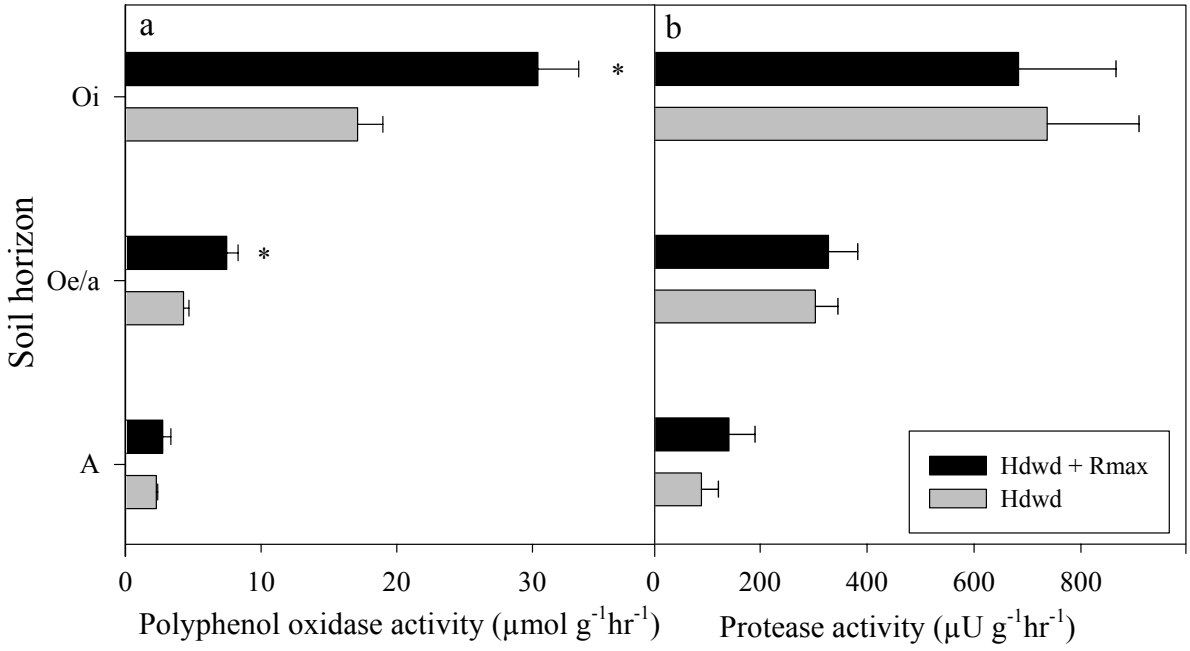
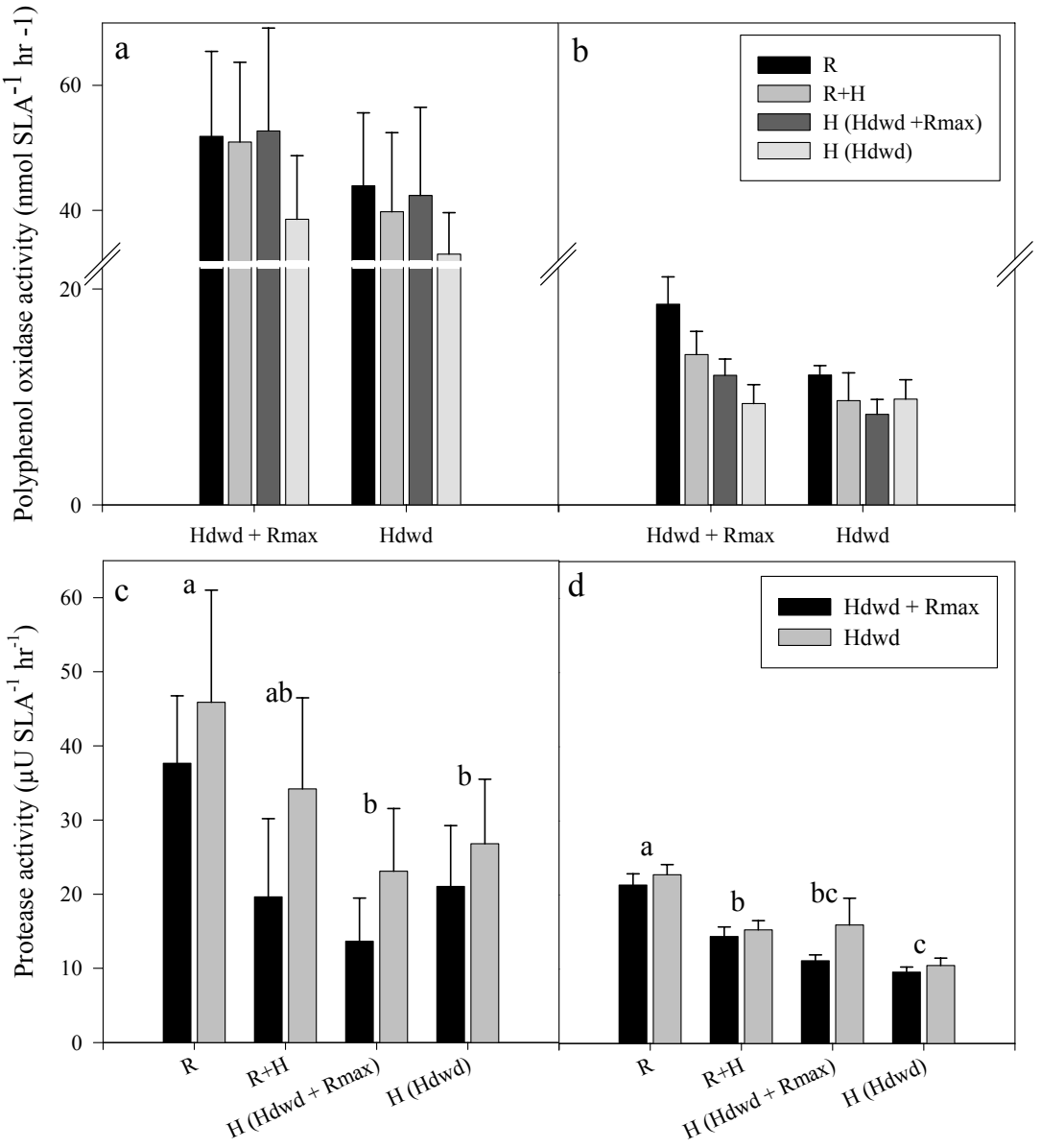


Figure 2.4. Extracellular enzyme activities on reciprocally placed leaf litter in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) for a) polyphenol oxidase activity, 10 months, b) polyphenol oxidase activity, 21 months, c) protease activity, 10 months and d) protease activity, 21 months. Significant differences ($\alpha < 0.05$) in protease activity among litter types denoted by different letters; $n = 4$ sampling blocks. Leaf litter treatments are as follows: R = *R. maximum* leaf litter only, R+H = *R. maximum* and hardwood species' leaf litter, H (Hdwd + Rmax) = hardwood species' leaf litter from Hdwd + Rmax plots, and H (Hdwd) = hardwood species' leaf litter from Hdwd plots.


a
b
c
d
Polyphenol oxidase activity (nmol SLA-1 hr -1)
Protease activity (µU SLA-1 hr-1)
R
R+H
H (Hdwd +Rmax)
H (Hdwd)
Hdwd + Rmax
Hdwd
0
20
40
60
10
30
50
ab
bc
c
R
R+H
H (Hdwd + Rmax)
H (Hdwd)

CHAPTER 3

THE FATE OF PROTEIN-TANNIN-N IN A TEMPERATE HARDWOOD FOREST: EVIDENCE OF A PLANT-SOIL-MYCORRHIZA FEEDBACK[1]

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ABSTRACT

Ericaceous plant litter polyphenols influence the N cycle of terrestrial ecosystems through the formation of protein-tannin complexes. The accumulation of protein-tannin N in soils, along with the greater ability of the ericoid mycorrhizal fungi of ericaceous plants to access protein-tannin N compared to ectomycorrhizal and arbuscular mycorrhizal fungi, is a hypothesized plant-soil-mycorrhiza feedback. We examined this hypothesis in a southern Appalachian hardwood forest with the understory shrub *Rhododendron maximum*. In forest microsites with and without *R. maximum*, we examined the fate of reciprocally placed ^{15}N enriched protein-tannin complexes. Based upon recovery of ^{15}N in soils, dissolved N pools and microbial biomass, protein-tannin complexes derived from *R. maximum* leaf litter were more recalcitrant than those derived from hardwood leaf-litter, especially when placed in hardwood soils. *R. maximum* ericoid mycorrhizal roots were more enriched than arbuscular and ectomycorrhizal roots of forest tress, particularly with *R. maximum* derived P-T complexes, suggesting that *R. maximum* has greater access to the organic N complexed by its own litter tannins compared to forest trees. Relative root recovery of protein-tannin N was greater for ericoid and ectomycorrhizal roots compared to arbuscular mycorrhizal roots per unit area under *R. maximum*. Less than 5 percent of the applied protein-tannin N was acquired by microbial biomass and mycorrhizal roots after 3 months, suggesting that these complexes have a long lasting affect on the N cycle of these temperate hardwood forests. The nutrient conserving habit of *R. maximum*, along with this observed plant-soil-mycorrhizal feedback with the formation and acquisition of recalcitrant organic N, may contribute to both the expansion of this shrub and the concomitant suppression of other plant species in southern Appalachian forests.

INTRODUCTION

Plants create ecosystem nutrient feedbacks through their litter quality and its alteration of the soil environment (Ehrenfeld et al., 2005; Chapman et al., 2006). Plant litter polyphenols (e.g. tannins) influence soil N availability and ecosystem N cycling (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003b), and polyphenolic concentrations of plant litter are often negatively correlated with soil fertility or forest productivity (Nicolai, 1988; Northup et al., 1995; Côté, 2000). The formation of polyphenol-organic N (protein-tannin, P-T) complexes is a proposed mechanism behind these patterns. P-T complexes decrease protein and plant material decomposition rates (Basaraba and Starkey, 1966; Benoit et al., 1968), resulting in organic matter accumulation (Handley, 1961) and lower rates of N mineralization (Bradley et al., 2000; Fierer et al., 2001; Castells et al., 2003; Kraus et al., 2004a).

The accumulation of P-T complexes in soils and the resulting decrease in soil N availability can impact plant community composition and structure. For example, ericaceous plants produce tannin-rich litter (Gallet and Lebreton, 1995; Preston, 1999), which stimulates P-T complex formation and lowers N availability in soils (Northup et al., 1998; Preston, 1999; DeLuca et al., 2002; Mallik, 2003). These patterns in N availability may be detrimental to ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) plants that rely upon the availability of inorganic N and cannot access recalcitrant P-T complex N. However, the ericoid mycorrhizal (ERM) fungi of ericaceous plants can degrade and access P-T complex N (Bending and Read, 1996a; Bending and Read, 1996b; Bending and Read, 1997; Read et al., 2004). Therefore, while altering patterns of N availability for other plant species, ericaceous plants may access P-T complex N through their ERM fungal symbionts, a hypothesized plant-soil-mycorrhizal N feedback (Northup et al., 1998). However, there is no unequivocal evidence in nature that

ericaceous, phenolic-rich plants can better access P-T complex N compared to other plants in their community (Hättenschwiler and Vitousek, 2000).

In the southern Appalachians, *Rhododendron maximum* L. an evergreen ericaceous shrub, forms dense thickets that alter forest community composition and structure (Baker and Van Lear, 1998; Lambers and Clark, 2003). *R. maximum* thickets increase leaf and root litter inputs and organic N storage in soils in hardwood forests, while decreasing the availability of inorganic N (Wurzburger and Hendrick, 2007). *R. maximum* litter contains a high concentrations of tannins (Hunter et al., 2003) and these tannins have a greater capacity to create P-T complexes in laboratory assays compared to tannins from hardwood trees (Wurzburger and Hendrick, 2007). These results suggest that the formation of P-T complexes contributes to organic N accumulation in *R. maximum* soils. P-T complex-N may be relatively unavailable to ECM and AM plants and trees, but available to *R. maximum* itself via ERM fungi. Lower inorganic N availability may, in part, be responsible for the observed patterns in seedling suppression and plant community composition in southern Appalachian forests. However, a direct examination of the fate of P-T complexes N in soils and mycorrhizal roots is necessary to provide evidence of a N feedback between *R. maximum* litter and ERM roots.

We conducted a field tracer study using ^{15}N labeled P-T complexes to quantify movement of ^{15}N into soil pools and mycorrhizal roots in a temperate hardwood forest. Our first objective was to examine the degradation of P-T complexes derived from tannins of hardwood leaf-litter and *R. maximum* leaf litter. We hypothesized that P-T complexes derived from hardwood litter tannins would be less recalcitrant than those derived from *R. maximum* because of the dramatic difference in O horizon formation between these two forest microsites (Boettcher and Kalisz, 1990; Wurzburger and Hendrick, 2007). Therefore, we predicted that more N from

hardwood P-T complexes would move into dissolved N pools, microbial biomass and mycorrhizal roots compared to *R. maximum* complexes. Our second objective was to compare P-T complex degradation between forest microsites with and without *R. maximum*. Since extracellular polyphenol oxidases are greater in *R. maximum* soils (Wurzburger and Hendrick, 2007) and because ERM fungi are present in these soils, we hypothesized that P-T complexes would degrade more rapidly in this forest microsite. Our third objective was to compare the relative differences among mycorrhizal roots in the acquisition of P-T complex N. We hypothesized that ERM roots would capture more P-T complex N compared to ECM and AM roots because of the documented saprotrophic activities of ERM fungi (Read et al., 2004). Greater enrichment of ERM roots relative to ECM and AM roots also provides evidence of a plant-soil-mycorrhiza feedback.

MATERIALS AND METHODS

Study location

Research was conducted along the boundary of the Coweeta Hydrologic Lab and the Nantahala National Forest, in western North Carolina, USA. Across a ~1 km transect at 1450m, traversing the NW-facing slope of Big Butt, we established 4 blocks of paired 4m x 4m plots in mature hardwood forests with and without a *R. maximum* understory (Hdwd+Rmax and Hdwd plots respectively). The forest overstory consists of ECM hosts *Quercus rubra* L., *Betula alleghaniensis* Britt., and AM host *Acer rubrum* L. The Hdwd plot understory is dominated by AM hosts *Acer pennsylvanicum* L. and *Amelanchier arborea* (Michaux f.) Fernald, and ECM host *Castanea dentata* (Marshall) Borkh. Annual herbs, ferns and sedges (AM and nonmycorrhizal, NM) are also present in the Hdwd plots. *R. maximum* is the only ERM host

present in these forest plots. Details of this forest community, soil characteristics, climate, leaf litter inputs and N cycling can be found elsewhere (Wurzburger and Hendrick, 2007).

Protein-tannin complex treatments

We reciprocally-placed P-T complexes in the two forest microsites by synthesizing P-T complexes with leaf litter tannins and a common protein source. Extracted soluble leaf litter tannins from the two microsites (Hdwd and Hdwd+Rmax) were combined with either ^{15}N enriched or unenriched protein, resulting in four treatments: ^{15}N-protein + Hdwd tannin, ^{15}N-protein + Hdwd+Rmax tannin, ^{14}N-protein + Hdwd tannin, and ^{14}N-protein + Hdwd+Rmax tannin (with the latter two being control treatments for the previous two). For clarity, plots in each of the forest microsites will be referred to as Hdwd and Hdwd+Rmax, while the synthesized P-T complexes will be referred to as H and H+R complexes based upon the origin of the tannins in the complex.

To sample tannins for the synthesis of P-T complexes, leaf litter was collected from four sampling blocks in fall, mixed by microsite, dried and ball-mill ground into a fine powder. Tannins were extracted from the ground tissues at room temperature with 50% methanol (0.1 g tissue ml^{-1} methanol) for 1 hr. The supernatant was separated from tissue by centrifugation and decanting. We grew *Ustilago maydis* (Persoon) Roussel, a rapidly growing basidiomycete, in liquid culture to create an enriched and unenriched protein source. Cultures grew in 2% glucose and salt solution of modified Melin-Norkrans media (Marx 1969) with 0.5% N applied as either $^{15}NH_4SO_4$ or $^{14}NH_4SO_4$, pH 7.0 at 29ºC and 150 rpm. After 48 hrs, fungal biomass was centrifuged, rinsed with deionized water, frozen and lyophilized for 24 hrs. Tissues were ground and extracted for protein with Tris-Glycine buffer (25 mM Tris base, 250 mM glycine, 1% SDS) (~ 500 mg tissue ml^{-1} buffer). Samples were centrifuged at 8,000 rpm for 40 min at 4ºC. The

protein extracts were mixed with tannin extracts at a 1:1 ratio, acidified with drops of 1M HCl to induce precipitation, and stored at 4ºC for 24 hrs. The resulting precipitates were centrifuged, rinsed with distilled water, frozen at 20ºC and lyophilized. The four P-T complex treatments all contained 52% C, but varied in percent N. H complexes contained 6.1% N, and H+R complexes contained 5.2% N. Atom percent enrichment of the H and H+R complexes were as follows: unenriched 0.39% and 0.38%; enriched 32.7 and 43.2%, respectively.

Application of treatments

The four treatments were applied once in each plot across the four blocks and sampled on two dates (3 mo and 1 yr after the application of the tracer). Eight PVC rings (1 cm high, area = 0.00204 m^2) were randomly positioned in each of the 8 plots, pinned into place with flagging wire at the surface of the Oe horizon. The PVC rings allowed for guidance of tracer application and subsequent core retrieval, but did not disturb the soil or restrict the growth of roots or fungal hyphae and the movement of the tracer. In August 2005, P-T complex treatments were ground, homogenized, suspended in deionized water and injected with 10 ml H_2O with a syringe into the top 0–5 cm of soil within the PVC ring. An additional 5 ml deionized H_2O was used to rinse the inside of the syringe and the remaining solution applied directly to the soil surface within the ring. For all treatments, N was applied in the form of P-T complexes at a rate of 0.24 g N m^{-2} which is approximately half the estimated annual N inputs resulting from *R. maximum* leaf-fall in the Hdwd+Rmax forest microsite (Wurzburger and Hendrick, 2007).

Soil sampling and extractions

Soils were sampled at two intervals; 3 mo and 1 yr after the tracer application with a corer of the same diameter as the PVC ring to a depth of 10 cm. Soil cores were placed on ice, transported to the laboratory and immediately processed. Care was taken to prevent cross-contamination of ^{15}N

between samples. Soil cores were sieved (4 mm) to separate roots from soil. Approximately 10-20g FW was extracted with 80ml 0.5M K_2SO_4 for dissolved N. An additional 5-10g FW soil was extracted with 40ml K_2SO_4 and 0.5ml $CHCl_3$ for extraction of labile microbial N (LMN) using an extraction method developed by Fierer and Schimel (2003). Briefly, samples (and appropriate controls) were shaken at 150 rpm at room temperature for 3 hrs, and gravity filtered with Whatman No. 1 filters (Whatman, Maidstone, UK) and extracts were bubbled with house air to remove any remaining $CHCl_3$. From the first set of extractions inorganic N (NH_4^+ and NO_3^-) was measured using a continuous flow colorimetric assay (Technicon AutoAnalyzer). Total dissolved N (TDN) was determined via persulfate digestion (Cabrera and Beare 1993) from the first sets of extracts, while microbial TDN (TDNm) was determined from the second set of extracts. Dissolved organic N (DON) was calculated through subtraction of DIN from TDN (DON=TDN-DIN), and LMN determined from subtraction of TDN from TDNm (LMN= TDNm-TDN). Remaining soils were dried at 60ºC, weighed to determine moisture content, ground and measured for percent C and N and N isotopes.

The ^{15}N of soil N pools (DIN, TDN and TDNm) were analyzed after diffusion into pairs of glass filter disks (Whatman GF/D, previous baked in muffle furnace at 500ºC for 4 hrs), acidified with 35µl 2M H_2SO_4 and wrapped in teflon tape following the methods of Stark and Hart (1996). For DIN extracts, KCl was added (to reach 1.5M) to raise the ionic strength of the solution, along with MgO and Devarda's alloy (-100 mesh, Sigma-Aldrich, Milwaukee, WI). For the persulfate digests, 270µl 10M NaOH was added to raise the pH of solutions. Blank samples were used to account for any background contamination of N for all soil extractions and N diffusions. Filter packets were dried for at least 48 hrs in a dessicator with concentrated H_2SO_4,

then wrapped in silver capsules (Costech Analytical Technologies Inc., Valencia, CA) and analyzed for total N and ^{15}N.

Mycorrhizal root sampling

Mycorrhizal roots (≤0.5 mm diam) from each entire soil core were sorted from remaining soil and organic matter and categorized by mycorrhizal type. Roots were sorted based upon anatomical characters of the host plant roots and mycorrhizal type under a dissecting microscope. Hdwd plots contained ECM and AM hosts, and uncommon, NM hosts (*Carex* spp.; Miller et al., 1999), while Hdwd+Rmax plots contained ERM, ECM and AM hosts. Dead roots and roots >0.5 mm in diameter were all excluded from the sampling.

ECM roots (typically 0.2-0.3 mm diam) were distinguished by root branching patterns and the presence of fungal mantles (Agerer, 1991). ERM hair roots of *R. maximum* roots were distinguished based upon their root diameter (0.1 mm) and a subset of these were verified under magnification to contain hyphal coils within cortical root cells (Read, 1996). AM roots of *Acer* spp and herbs were slightly larger in diameter (0.3-0.5 mm), transparent and easily distinguished from other mycorrhizal roots. A subsample of AM roots were visually inspected for arbuscules (Wurzburger and Bledsoe, 2001). Since *Carex* spp. were relatively uncommon, and have been reported as both NM and AM hosts (Miller et al., 1999) these root types were combined in sampling. All roots were washed in deionized H_2O, brushed to remove adhering organic matter and soaked in 0.5mM $CaCl_2$ to remove any apoplastic or externally bound ^{15}N (Näsholm et al., 1998) and rinsed again in H_2O. Roots were dried, weighed, ground to a fine powder and analyzed for percent N and ^{15}N.

^{15}N analysis and calculations

C, N and ^{15}N was analyzed from ground soil and mycorrhizal roots and glass filters with a isotope ratio mass spectrometer at the Stable Isotope Facility at the Institute of Ecology, University of Georgia. For all enriched samples, atom percent enrichment (APE) was calculated with Equation 1, with pairs of enriched and unenriched samples where S indicates the enriched sample and C is the control sample.

$$APE = [(^{15}N \text{ atoms/ total N atoms})_S - (^{15}N \text{ atoms/ total N atoms})_C] * 100 \quad [1]$$

Percent recovery of ^{15}N involved multiplying the APE of a pool by the total N of the pool, and then accounting for the portion of undetected ^{14}N associated with the applied treatments from their original atom percent. The APE of DIN, TDN and TDNm were determined directly, while the APE of DON and LMN were calculated from Equations 2 and 3, where AP is the atom percent of ^{15}N and C is the concentration of N.

$$APE_{DON} = [(AP_{TDN} * C_{TDN}) - (AP_{DIN} * C_{DIN})]/ [C_{DON}] \quad [2]$$

$$APE_{LMN} = [(AP_{TDNm} * C_{TDNm}) - (AP_{TDN} * C_{TDN})]/ [C_{LMN}] \quad [3]$$

Statistical analyses

All data were tested for normality, and transformed when needed. In general, we used a split-plot or a split-split-plot repeated measures ANOVA using SAS software (SAS Institute, Cary, NC). Block (n=4) was a random effect and incorporated into each model. Fixed effects included microsite (n=2), P-T complex type (n=2), time (n=2) and mycorrhizal root type (n=3). Specifically, a split-plot repeated measures design was used for soil N pools (total N, DIN, DON, and LMN) to examine the effects of microsite (whole-plot factor) time (split-plot factor) on N pool size. A split-split plot repeated measures design was used to analyze the response of root biomass and root total N to microsite (whole-plot factor), time (split-plot factor) and mycorrhizal

root type (split-split-plot factor). Total ^{15}N soil and total root ^{15}N recovery were analyzed with a repeated measures split-split-plot ANOVA, in response to microsite (whole-plot factor), time (split-plot factor), and complex type (split-split-plot factor). For root APE of ^{15}N we used a split-split-plot design, with P-T complex type as the whole plot factor, time as the split-plot factor, and mycorrhizal root type the split-split-plot factor. For all analyses, we performed a *post hoc* least square means separation technique to determine significant differences ($\alpha<0.05$) among all factor combinations.

RESULTS

Soil pools and mycorrhizal roots

Total soil N differed by forest microsite ($P= 0.0003$) and time of sampling ($P<0.0001$). Percent N was greater in the Hdwd+Rmax plots than Hdwd plots at both sampling times, percent N was greater in both microsites at the 1 yr sampling compared to the 3 mo sampling (Table 3.1). Total DIN displayed a microsite by time interaction ($P=0.0027$), with greater DIN concentrations in the Hdwd+Rmax microsite at the 1 yr sampling. DON also displayed a microsite by time interaction ($P=0.05$) with greater DON concentrations at the 3 mo sampling compared to 1 yr, but only in the Hdwd microsite. LMN was greater in the Hdwd+Rmax microsite at both sampling times ($P=0.0008$) (Table 3.1).

Total mycorrhizal root biomass was greater in the Hdwd+Rmax plots compared to Hdwd plots by 54 and 20% at the 3 mo and 1 yr sampling, respectively. Mycorrhizal root biomass responded to a microsite by root type interaction ($P=0.0021$). In general, in the Hdwd+Rmax plots, AM root biomass was lower than ERM and ECM root biomass at both dates, while ECM and ERM root biomass were not different from one another. In the Hdwd plots, there was no difference between ECM and AM root biomass, although ECM root biomass increased from the

3 mo to 1 yr sampling dates. Between plot types, ECM and AM root biomass were not significantly different from each other (Table 3.1). Between microsites and sampling dates, mycorrhizal root percent N differed by root type (P<0.0001) with generally greater percent N in AM and ECM roots compared to ERM roots (Table 3.1).

Total soil ^{15}N recovery

Total soil ^{15}N recovery was related to P-T complex type (P=0.0001), with consistently greater recovery from H+R complexes compared to H complexes regardless of sampling time or microsite. There was no overall response to microsite, time or to an interaction of these factors, however at the 1 yr sampling, there was greater recovery of ^{15}N from H+R complexes in Hdwd soils compared to Hdwd+Rmax soils (Figure 3.2).

^{15}N detection in dissolved N pools and microbial biomass

At the 3 mo sampling, enrichment of ^{15}N in DIN responded to complex type (P=0.0047), with greater enrichment in soils treated with H complexes compared to H+R complexes (not shown). Recovery of ^{15}N in DIN pools was related to complex type (P=0.0069) and microsite (P=0.0098), with greater ^{15}N recovery from H complexes in the Hdwd microsite compared to the other treatments (Figure 3.3a). Enrichment of soil DON was not significantly different among complex types and forest microsites (data not shown). ^{15}N recovery in DON was significantly related to complex type (P=0.042). In general, ^{15}N recovery in DON was greater for H complexes compared to H+R complexes, but this was significant only in the Hdwd microsite (Figure 3.3a). LMN enrichment of ^{15}N was greater in soils treated with H complexes compared to H+R complexes (P=0.07) (not shown). LMN recovery of ^{15}N was significantly related to an interaction between complex type and microsite (P=0.035). In the Hdwd microsite, there was greater ^{15}N recovery in LMN from H complexes compared to H+R complexes, but there were no

differences between complex types in LMN ^{15}N recovery in the Hdwd +Rmax microsite (Figure 3.3a). All ^{15}N data from dissolved soil pools and microbial biomass from the 1 yr sampling date were lost due to failure of the isotope ratio mass spectrometer.

Root enrichment and recovery of ^{15}N

Because of variations in total mycorrhizal root biomass and root biomass by mycorrhizal type, we analyzed root ^{15}N data in three ways. First, total root recovery per soil core gave the perspective of total root acquired ^{15}N per unit area between the forest microsites. Second, since the biomass and percent N differed among mycorrhizal root types and by time and location, we analyzed the APE of individual mycorrhizal root types to allow for a normalized view of ^{15}N acquisition and the impact of ^{15}N among mycorrhizal root pools. Third, relative recovery of ^{15}N among root types revealed the percentage of total root acquired N associated with each mycorrhizal root type. Relative recovery is related to both biomass and APE, and gives the perspective of the relative acquisition of ^{15}N to mycorrhizal roots per unit area of the forest floor.

Total mycorrhizal root recovery of ^{15}N had a significant time by microsite interaction (P=0.028), with greater root recovery in the Hdwd+Rmax plots than Hdwd plots at the 3 mo sampling and no difference between the two microsites at the 1 yr sampling. Total ^{15}N recovery decreased by ~ 50% from the 3 mo to the 1 yr sampling in both microsites (Figure 3.3a and 3.3b).

^{15}N enrichment (APE) of mycorrhizal roots differed by sampling times (P<0.0001) and also differed by mycorrhizal root type (P=0.0021). Root enrichment decreased from the 3 mo to the 1 yr sampling date. In general, ERM roots were more enriched relative to ECM and/or AM roots but this pattern differed by P-T complex type. ERM roots were more enriched than ECM and AM roots with the H+R complex, while ERM roots were more enriched than ECM roots

only with the H complex (Figure 3.4a). At the individual sampling dates, although the APE of roots did not have an overall response to complex type, there were differences among root types between the P-T complex types at the 3 mo sampling date (P=0.063). Here ERM roots were generally the most enriched among root types, however, this was only significant with the H+R complex type. At the 1 yr sampling, root APE values were moderately greater in ERM roots than the other root types (P=0.08) (data not shown).

The relative recovery of ^{15}N among root types was related to microsite (P=0.02) and root type (P<0.0001) with no significant effect of time or P-T complex type. In Hdwd+Rmax plots, ERM and ECM roots recovered a similar percentage of ^{15}N, and both recovered more ^{15}N than AM roots. In the Hdwd plots, the percentage of ECM root recovery was greater than that of AM roots (Figure 3.4b).

DISCUSSION

Protein-tannin N

Plant litter polyphenols can depress decomposition and N mineralizaton rates in forest ecosystems (Northup et al., 1998; Preston, 1999; DeLuca et al., 2002) and lead to decreases in forest productivity (Côté, 2000; Nilsson and Wardle, 2005). In extreme cases, the alteration of the N cycle by litter polyphenols can contribute to the suppression of forest regeneration, as observed with ericaceous species in forests world-wide (Mallik, 2003). Despite the importance of litter polyphenols on ecosystem function, we know relatively little about P-T interactions and the fate and degradation of soil P-T complexes *in situ* (Hättenschwiler and Vitousek, 2000; Lovett et al., 2004). We used a temperate hardwood forest in the southern Appalachians as a model system to study the importance of *R. maximum* litter tannins on N cycling. Leaf and root litter tannins from *R. maximum* microsites precipitate nearly twice the amount of protein per unit litter mass

than those from hardwood trees (Wurzburger and Hendrick, 2007). This observation, together with approximately 40% greater root and leaf litter inputs in this microsite (Wurzburger and Hendrick, 2007), suggests that P-T complexes are more likely to form in these soils. Based upon levels of soil ^{15}N recovered in this study, P-T complexes derived from *R. maximum* leaf litter display greater recalcitrance than their hardwood counterparts. Therefore, P-T complex formation and accumulation in *R. maximum* soils may explain the high mass and N content of the organic horizons in this forest microsite (Boettcher and Kalisz, 1990; Wurzburger and Hendrick, 2007).

We utilized free soil *in-situ* incubations of ^{15}N enriched P-T complexes and a low rate of N application to better understand the fate of P-T complex N in soil biotic and abiotic pools. With minimal disturbance of roots and soils and low levels of N application we could, with greater confidence, attribute patterns in the degradation and movement of ^{15}N to functional differences between P-T complex types, soil microbial communities and mycorrhizal roots. One limitation of this method was our inability to account for the entire pool of applied ^{15}N. However, our conclusions about complex degradation were taken from recovery of ^{15}N in soil pools, microbial biomass and mycorrhizal roots within the applied soil volume, which we believe is likely to represent patterns of the remaining undetected ^{15}N outside the sampling volume.

Protein-tannin N and the N cycle

P-T complexes undergo several stages of degradation before the N is biologically available (Figure 3.1). Although direct complex degradation by extracellular enzymes has not been demonstrated in the literature, enzymes such as polyphenol oxidases may release protein from P-T complexes (Burke and Cairney, 2002). Proteins or peptides associated with the DON pool can be further degraded by proteolytic enzymes, and the resulting amino acids can be taking up by

plants or microbes (Read et al., 2004). Enzymes are produced by bacteria or fungi (saprobes or mycorrhizal symbionts) and these transformations can occur in the soil solution or on root or hyphal surfaces. Once bioavailable, P-T complex N has many potential fates. If taken up by microbes, the N can be subsequently released as mineral N and then acquired by plants. Alternatively, P-T complex N degraded into amino-acid form can be directly acquired by mycorrhizal fungi and plants, bypassing microbial transformations of N (Northup et al, 1998). Our methods cannot reveal the precise pathway by which P-T complex-N became incorporated into mycorrhizal roots. Regardless of the pathway, the accumulation of P-T complex N into mycorrhizal root or plant biomass will determine its role in plant nutrition and the N cycle. If root fungi or rhizosphere fungi and bacteria are directly contributing to the degradation of P-T complexes, we expect direct movement of P-T complex N into those root tissues.

P-T complexes derived from *R. maximum* microsite leaf litter tannins were more recalcitrant than those derived from hardwood leaf litter tannins. This was supported by greater total soil recovery of ^{15}N from H+R complex compared to H complexes and patterns in the recovery of ^{15}N in DIN, DON and LMN. We recovered the most ^{15}N in DIN in Hdwd soils treated with H complexes, and the least recovered from Hdwd soils treated with H+R complexes. A similar pattern held for ^{15}N recovery in DON and LMN. The differences in ^{15}N recovery between H and H+R complexes was only apparent in the Hdwd microsite, suggesting that this soil microbial community is not well adapted to degrading P-T complexes derived from *R. maximum*. In contrast to soil N pools, total mycorrhizal root recovery of ^{15}N was related to microsite, with greater recovery in Hdwd+Rmax microsites compared to Hdwd microsites. This pattern was due to both greater mycorrhizal root biomass in this microsite as well as the presence of ERM roots with a relatively high ability to acquire P-T complex N among the mycorrhizal

root types (see below). Recovery of ^{15}N from microbial biomass and total mycorrhizal roots was more similar in the Hdwd+Rmax compared to the Hdwd microsite suggesting that mycorrhizal roots in these soils are degrading and accessing P-T complex N to a similar extent as are soil microbes. Although a greater amount of ^{15}N was captured in microbial biomass than in mycorrhizal roots in Hdwd soils, this N is may become available to plant roots over the long term. The release of this P-T complex-N as mineral N is dependant upon the turnover and N demands of the microbial community.

Root acquisition of protein-tannin N

We observed differences among mycorrhizal root types in the acquisition of P-T complex N, with greater ^{15}N enrichment of ERM roots compared to ECM and AM roots, but this pattern was only significant with the more recalcitrant, H+R complexes. These results support the hypothesis that ERM fungi and plants have greater access to recalcitrant forms of N compared to ECM and AM plants. Because all three mycorrhizal root types coexist and intermingle in these soils, we believe that these patterns in ^{15}N enrichment indicate physiological differences among mycorrhizal roots (and associated microbial assemblages) resulting in their differential ability to access soil N sources. In the field and in pot studies, ERM, ECM and AM plants all take up forms of inorganic and amino-acid N at relatively similar rates (Nordin et al., 2001; Persson et al., 2003; Bennett and Prescott, 2004; Rains and Bledsoe, 2007). However, the potential for ERM fungi to actively degrade and access recalcitrant N forms is an important consideration for plants in organic, nutrient poor soils (Read et al, 2004). In pure culture, ERM fungi can degrade and acquire N from protein, plant and fungal necromass and chitin (Leake and Read, 1991; Kerley and Read, 1995; Kerley and Read, 1998). However, pot studies of ERM, ECM and AM plant acquisition of complex organic N sources have produced mixed results (Wu et al., 2003;

Bennett and Prescott, 2004; Rains, 2004). To our knowledge, this study is the first test and confirmation of relative differences among mycorrhizal roots in the acquisition of P-T complex N *in- situ.*

Greater access to recalcitrant N substrates by ERM roots than by ECM and AM roots is likely the result of the attributes of the roots and mycorrhizal fungi. First, the saprotrophy of ERM fungi, including a tendency for extracellular enzyme production, contributes to the degradation of organic substrates (Read et al., 2004). Greater polyphenol oxidase activities in Hdwd+Rmax soils support the hypothesis that the ERM fungi and local microbial communities contribute to extracellular enzyme production (Wurzburger and Hendrick, 2007). Also, ERM roots of *R. maximum* associate with diverse fungi, a number of which are traditionally viewed as soil and litter saprotrophs (Wurzburger et al., Ch. 4). Loose associations between ERM roots and soil saprotrophic fungi may indirectly benefit the plant by increasing decomposition activities in the rhizosphere and hence increasing the availability of nutrients contained in recalcitrant organic substrates. Greater specific root length of ERM hair roots compared to ECM and AM roots may increase root exploratory area and interception of immobile soil organic substrates per unit root biomass (Wurzburger and Hendrick, 2007).

Relating patterns of mycorrhizal root enrichment to plant N acquisition at the stand level is important to understand the impact of P-T complex N on the N cycle. However, the contribution of P-T complex N to the N nutrition of individual forest trees and shrubs is not easily quantified because both mycorrhizal root biomass and N concentration differ among the mycorrhizal root types. Although ERM roots were more enriched in ^{15}N, the capture of ^{15}N per unit soil volume was similar between ERM and ECM roots, both of which recovered a greater amount of ^{15}N than AM roots. The similarity between ERM and ECM recovery of ^{15}N per unit

volume is related to both greater biomass (not significant) and greater N concentration in ECM roots than in ERM roots. In lower elevation mixed hardwood forests at Coweeta, *R. maximum* thickets contribute ~8% of aboveground net primary production (Monk et al., 1985; Monk and Day, 1985). However, because of slow leaf turnover and the low N concentration of leaves, the contribution of *R. maximum* to N associated in annual aboveground production is only 4% of the stand total (Day and Monk, 1977; Monk et al., 1985). Therefore, N demands are relatively low for *R. maximum* and similarities in N acquisition per unit area between ERM and ECM roots may create a greater impact on the N nutrition of the shrub than to ECM forest trees.

Mycorrhizal root enrichment decreased from the 3 mo to 1 yr sampling date and this difference in root enrichment could be related to patterns in root production. After the tracer was applied in August, the first sampling occurred in October when fine root production peaks in forests at Coweeta (Davis et al., 2004), and newly acquired N may have become associated with root biomass. The second sampling occurred in August of the following year, and a greater proportion of acquired N may have been translocated to aboveground tissues during the growing season. Total plant recovery of ^{15}N was likely much greater than what we observed in mycorrhizal roots, and we must acknowledge the possibility that AM, ECM and ERM plant hosts differentially allocated ^{15}N among above and belowground tissues during the study. However, fine root or mycorrhizal root enrichment of ^{15}N is typically correlated with total plant enrichment in tracer studies (Näsholm et al., 1998; Rains, 2004; Rains and Bledsoe, 2007), and therefore, may be a useful index of total plant acquisition when it is not feasible to harvest and detect ^{15}N in the aboveground biomass of forest shrubs and trees.

Turnover of protein-tannin N

The impact of P-T complexes on the N cycle has remained elusive because they are recalcitrant and difficult to study. In our study, after 3 mo of decomposition in the field, less than 5% of the applied ^{15}N in the form of P-T complexes (or 0.012g m^{-2}) was associated with microbial biomass and mycorrhizal roots. The formation and recalcitrance of P-T complexes may be a function of the composition, structure and stereochemistry of tannin molecules (Kraus et al., 2003a). In laboratory incubations, P-T complexes derived from various plant species displayed a range of half lives from 2 days to 56 weeks, and some with unpredictable half lives (Howard and Howard, 1993). In a pot study, 20 days after applying P-T complexes to soils, less than 2% of the protein-tannin-N was detected in plant biomass with no significant differences in ^{15}N recovery among ERM, ECM and AM plants (Bennett and Prescott, 2004). Microbial acquisition of P-T complex-N is similar to that of plants. In a temperate coniferous forest, less than 6% of applied P-T complex N was recovered in microbial biomass after 1 year of decomposition (Holub and Lajtha, 2004). These studies demonstrate the slow degradation of P-T complexes and their legacy in soils. In our study, total soil ^{15}N recovery changed only subtly from 3 mo to 1 yr suggesting that most of the degradation occurred within the initial 3 mo of the study. Similarly, the movement of ^{15}N into dissolved soil pools and microbial biomass occurred at a greater rate from 0-14 days than it did from 0-365 days in a previous study (Holub and Lajtha, 2004). P-T complexes formed from litter tannins are likely to vary in recalcitrance, a result of the chemistry of the organic N and the tannins. Rates of P-T complex degradation determined from month to year-long field studies are probably misleading if the data represent the more labile portion of this N pool. We need to more accurately determine the residence times of P-T complexes in order to understand their impact on forest N cycles.

Conclusions

Not only is P-T complex formation more likely to occur in *R. maximum* forest microsites than in hardwood microsites (Wurzburger and Hendrick, 2007), but the P-T complexes derived from the leaf litter of this microsite degrade more slowly. These results suggest that P-T complexes contribute to the accumulation of organic matter and the suppression of N mineralization in these soils. The soil microbial communities and mycorrhizal roots of the forest microsites differed in their relative ability to acquire P-T complex N. ERM roots of *R. maximum* acquired the most P-T complex N among mycorrhizal root types per unit biomass. The direct acquisition of P-T complex N by ERM roots of *R. maximum* may be an important contribution to its N nutrition, especially when considering the potential residence time of P-T complexes in soils and the N conserving habit of this shrub. A feedback between *R. maximum* litter and ERM fungi may contribute to the expansion of this shrub in southern Appalachian forests, while reductions in N availability may further suppress ECM and AM tree seedlings. We still need to understand the mechanisms of P-T complex formation and degradation in order to fully appreciate the role of litter tannins on the N cycle of forest ecosystems.

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Table 3.1 Soil N pools, mycorrhizal root biomass and total N in hardwood forest (Hdwd) microsites with and without *R. maximum* (Rmax) at the 3 month and 1 year sampling periods. Values are means and (SE) from $n = 4$ blocks of paired sampling plots. Letters denote significant differences ($\alpha < 0.05$) between sampling times and forest microsites for soil N variables, and between microsite, time and mycorrhizal root type for root biomass and percent N.

	Hdwd		Hdwd + Rmax	
	3 month	1 yr	3 month	1 yr
Soil N				
Total N (%)	0.43 0.02 c	0.66 0.03 b	0.59 0.04 b	0.93 0.10 a
DIN[a] (mg kg^{-1})	15.91 2.10 b	15.85 1.52 b	12.05 1.57 b	30.16 5.40 a
DON[a] (mg kg^{-1})	63.11 6.88 a	47.77 1.95 b	55.37 3.78 ab	57.18 5.78 ab
LMN[a] (mg kg^{-1})	46.00 5.03 bc	43.24 2.80 c	67.95 9.17 ab	96.45 15.7 a
Mycorrhizal root biomass (g m^{-2})				
AM[b] & NM[b]	31.99 9.29 bc	28.32 3.62 bcd	23.65 7.23 cd	12.42 2.45 d
ECM[b]	34.19 9.29 abc	59.37 14.10 ab	62.01 8.39 a	58.50 10.29 ab
ERM[b]			60.66 9.76 ab	40.03 8.10 ab
Mycorrhizal root N (%)				
AM & NM	1.45 0.14 b	1.42 0.05 b	1.79 0.07 a	1.55 0.05 ab
ECM	1.56 0.12 ab	1.58 0.05 ab	1.52 0.09 ab	1.74 0.06 a
ERM			1.34 0.08 c	1.42 0.03 b

[a] Soil N pools are dissolved inorganic N (DIN), dissolved organic N (DON),and labile microbial N (LMN).

[b] Mycorrhizal root types include arbuscular mycorrhizal (AM), nonmycorrhizal (NM), ectomycorrhizal (ECM) and ericoid mycorrhizal (ERM).

Figure 3.1 Hypothesized N cycle pathways of protein-tannin N and acquisition by mycorrhizal roots. N movement from protein-tannin complexes to dissolved organic N (DON), microbial N, dissolved inorganic N (DIN) and mycorrhizal roots (Myco Root N). Extracellular enzymes mediate the transformations of protein-tannin complex-N to DON and from DON into bioavailable form (e.g. amino-acid).

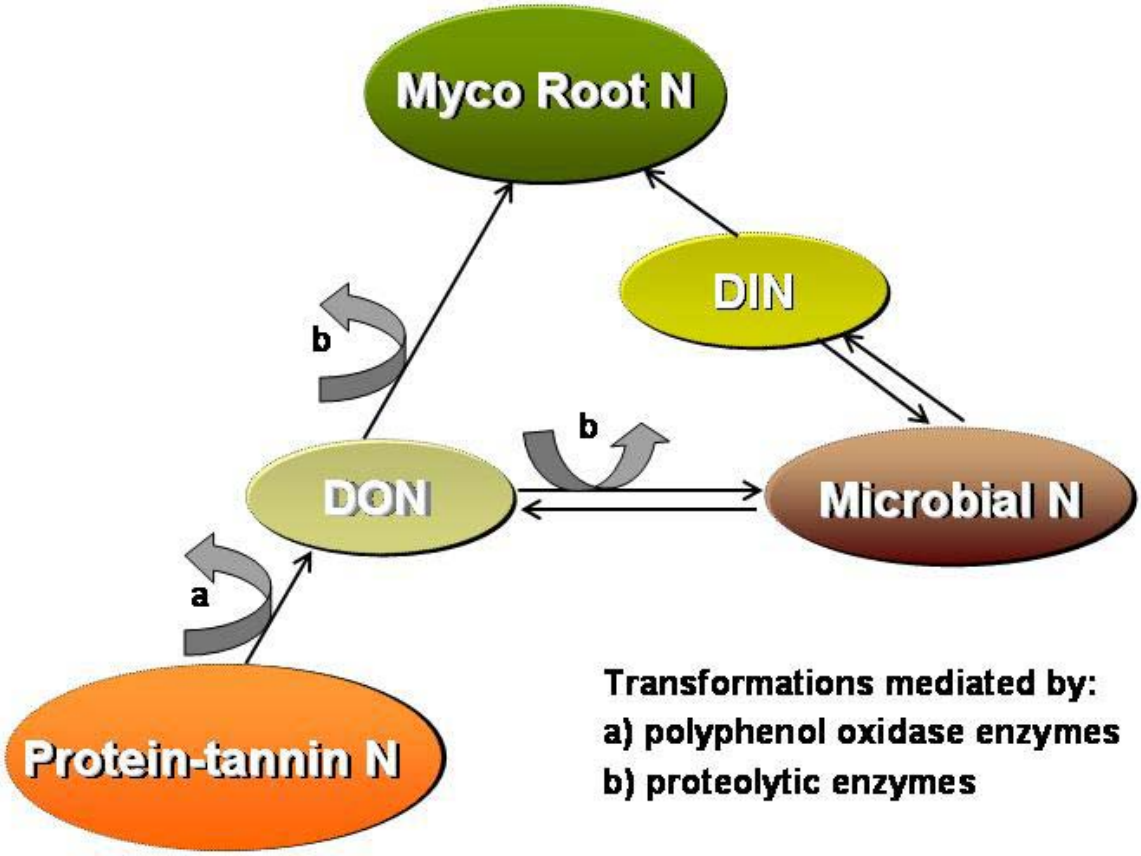


Figure 3.2 Total soil recovery of ^{15}N from H and H+R protein-tannin complexes applied to hardwood forest (Hdwd) microsites with and without *R. maximum* (Rmax) at the 3 month and 1 year sampling dates. Mean values ± SE bars from n = 4 blocks of paired sampling plots. Significant differences ($\alpha < 0.05$) between complex types and sampling times are indicated by different letters.

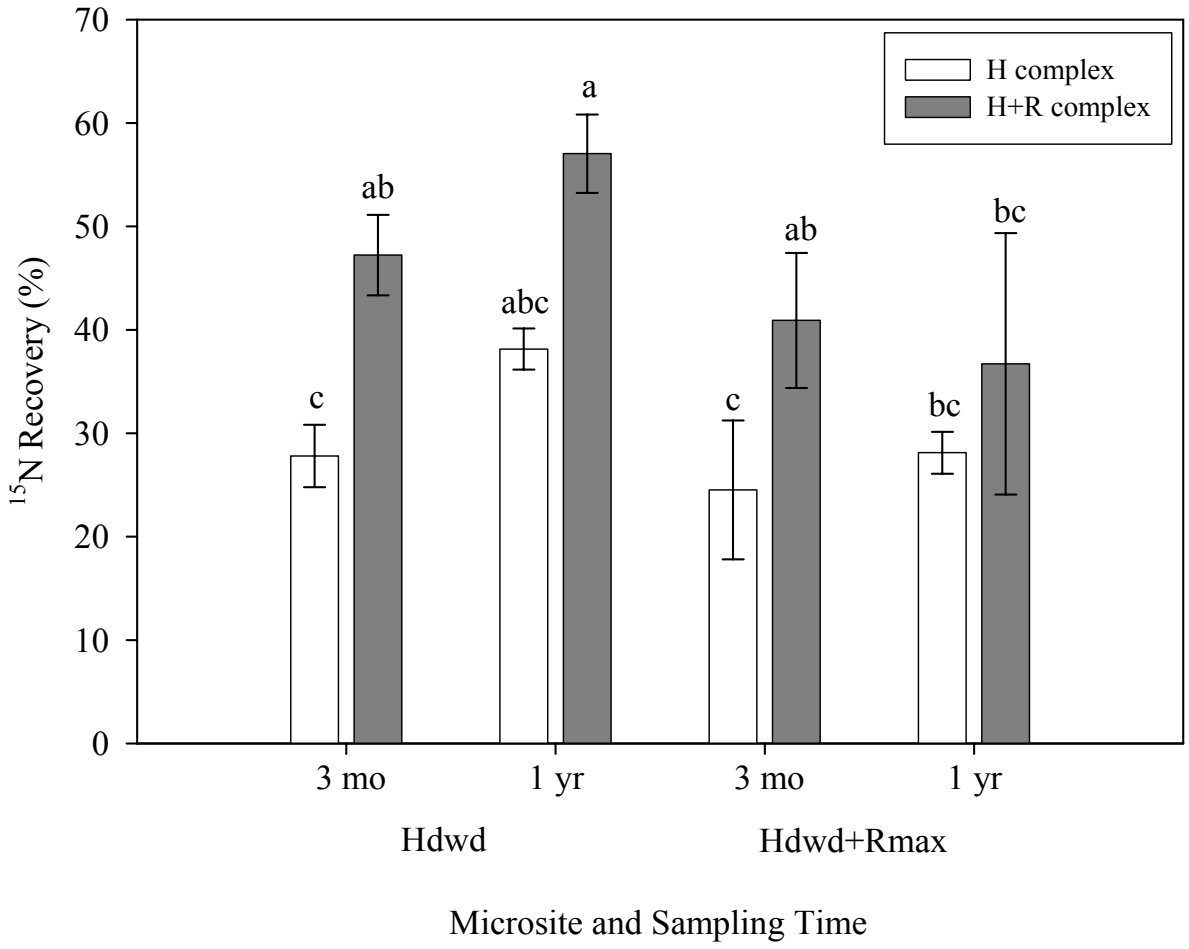
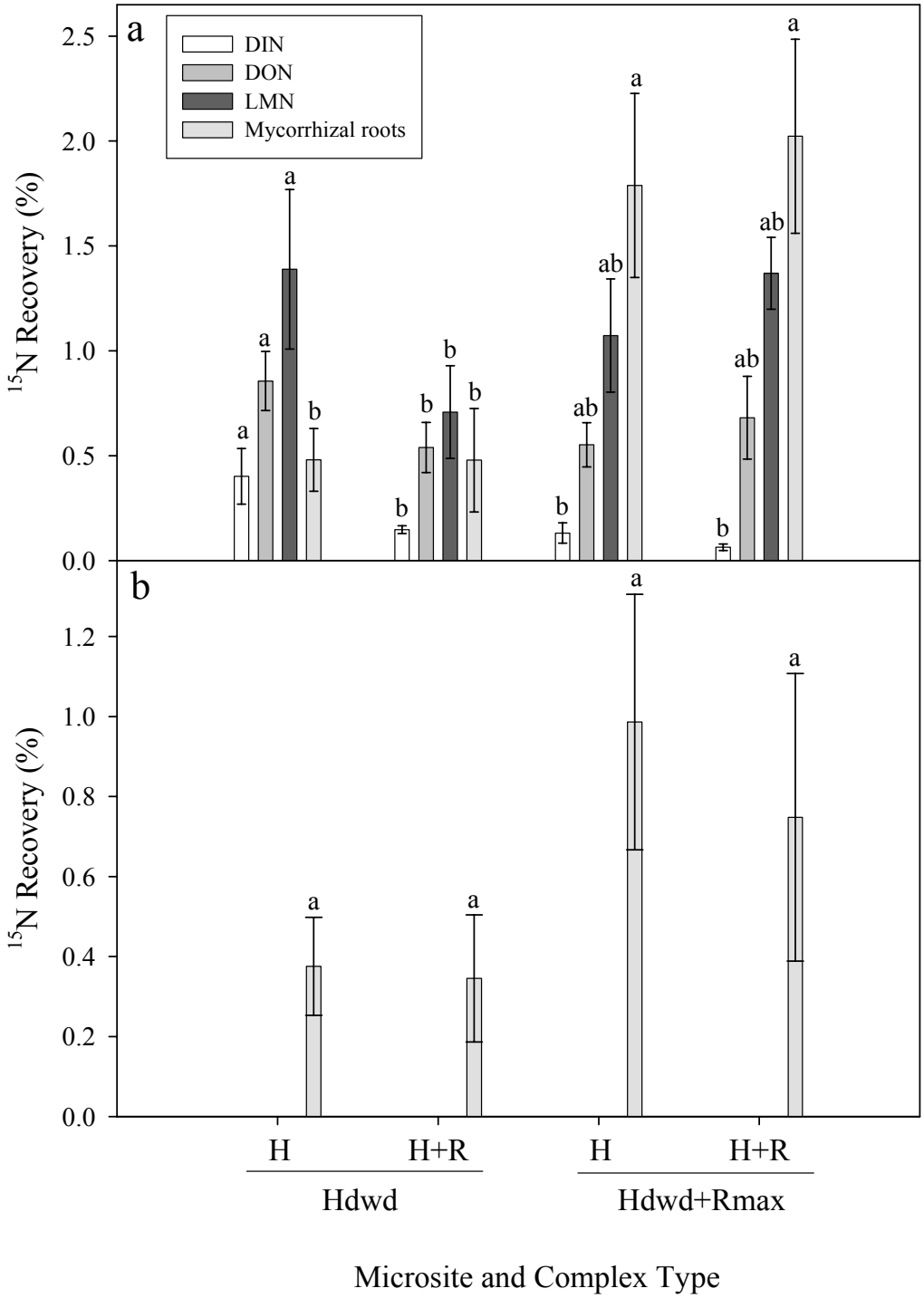
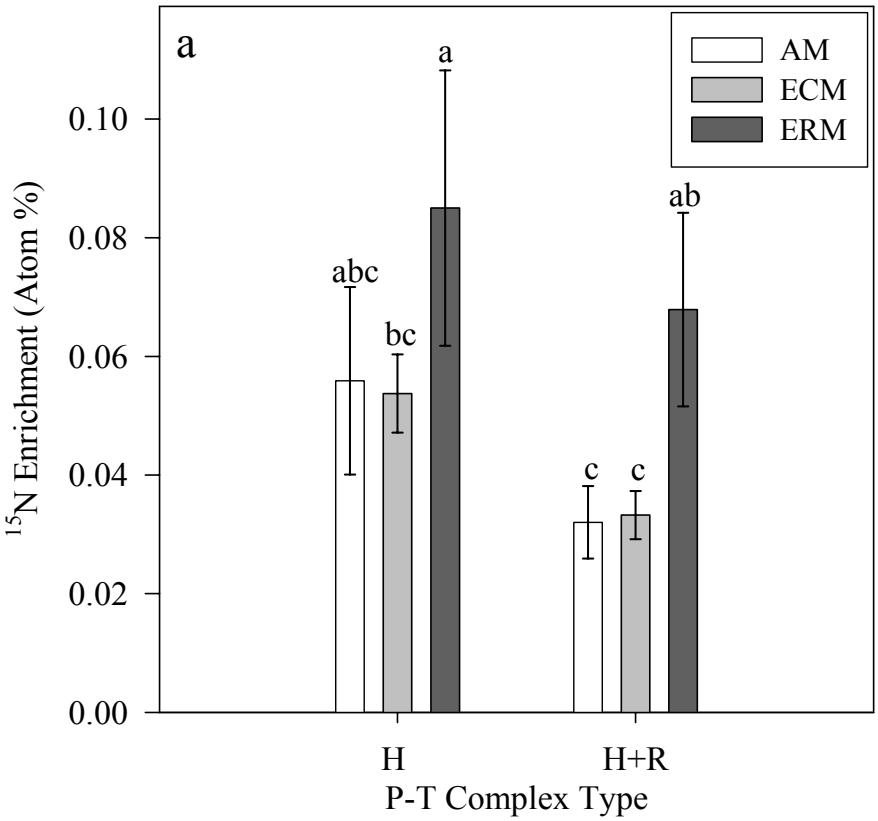


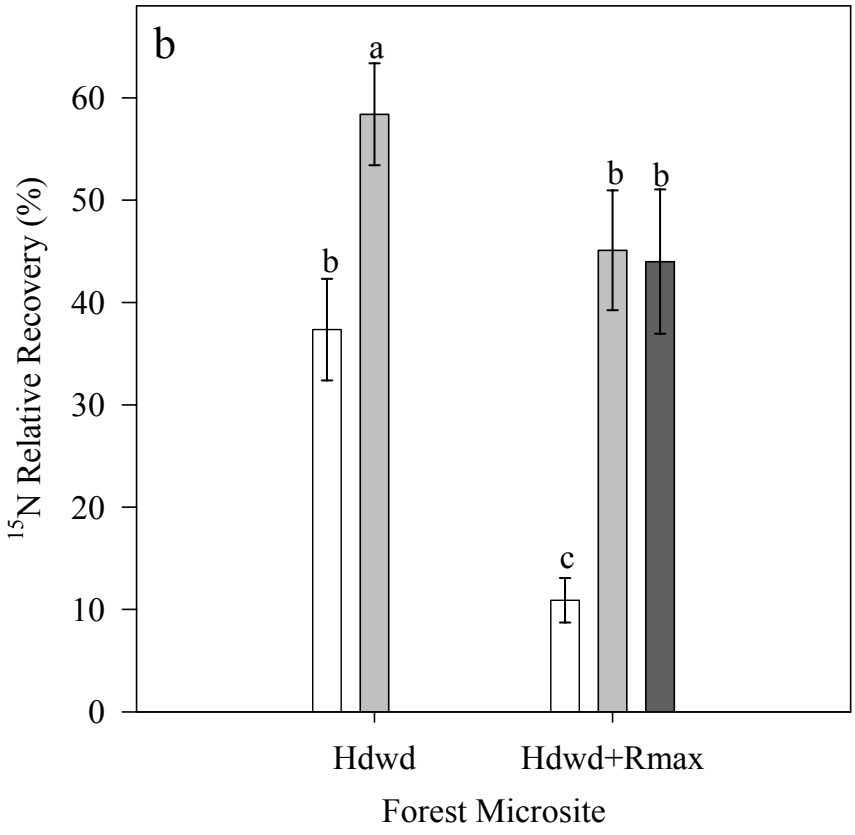
Figure 3.3 Recovery of ^{15}N from H and H+R protein-tannin complexes applied to hardwood forest (Hdwd) microsites with and without *R. maximum* (Rmax) in DIN, DON, LMN and total mycorrhizal roots at sampling times a) 3 month and b) 1 year. Mean values ± SE bars from $n = 4$ blocks of paired sampling plots. Significant differences ($\alpha < 0.05$) within an individual N pool between microsites and complex types at a given sampling time denoted by different letters.[a,b]



[a] Dissolved inorganic N (DIN), dissolved organic N (DON) and labile microbial N (LMN).
[b] Data for ^{15}N recovery in DIN, DON and LMN at the 1 year sampling were lost due to equipment failure.

Figure 3.4 Detection of ^{15}N from H and H+R protein-tannin complexes in mycorrhizal roots in hardwood forest (Hdwd) microsites with and without *R. maximum* (Rmax) expressed as a) ^{15}N enrichment and b) ^{15}N relative recovery. Mycorrhizal roots are ericoid mycorrhizal (ERM), ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM). Data pooled from both sampling dates ($n = 2$). Mean values ± SE bars from $n = 4$ blocks of paired sampling plots and significant differences ($\alpha < 0.05$) in enrichment and recovery among mycorrhizal root types and between complex types or forest microsites denoted by different letters.


a
AM
ECM
ERM
0.10
0.08
0.06
0.04
0.02
0.00
^{15}N Enrichment (Atom %)
abc
bc
a
c
c
ab
H
H+R
P-T Complex Type


b
60
50
40
30
20
10
0
^{15}N Relative Recovery (%)
b
a
c
b
b
Hdwd
Hdwd+Rmax
Forest Microsite

CHAPTER 4

DIVERSE FUNGI AND FUNGAL LACCASE GENE SEQUENCES ASSOCIATE WITH ERICOID MYCORRHIZAL ROOTS OF RHODODENDRON[1]

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ABSTRACT

Ericoid mycorrhizal fungi are considered important for the nutrient acquisition of ericaceous plants but the structure of their communities is poorly understood. We characterized the composition and structure of the ericoid mycorrhizal root fungal community of *Rhododendron maximum*, an understory shrub of southern Appalachian forests. We sampled ericoid mycorrhizal roots from organic and mineral soil horizons across four replicate sampling plots in hardwood forests along a high elevation ridge in western NC, USA. Using both a culture- and cloning-based approach (direct DNA extraction and amplification of the ITS region), we observed 71 putative fungal taxa from four fungal phyla based upon sequence similarity. Only three taxa were observed using both methods. Twenty two fungal taxa could not be identified beyond the Kingdom Fungi or a fungal phylum. These fungi include previously characterized ericoid symbionts *Rhizoscyphus ericae* and *Oidiodendron maius*, and several potential symbionts in the Helotiales, Chaetothryiales and Sebacinales. Other fungi included ectomycorrhizal taxa, saprotrophs and a plant pathogen. Based upon data obtained from cloning fungal ITS products, fungal richness was greater in roots from the O horizon compared to the A horizon, and the entire fungal community was characterized by several rare taxa. Nearly 70% of fungal ITS clones were each observed only once among sampled cores, and 50% were observed once from the clone collection. The production of extracellular enzymes, such as laccase, by ericoid mycorrhizal fungi, may aid in substrate degradation and plant nutrient acquisition in organic soils. We characterized a portion of the laccase gene sequence from cultured root fungi. Each of these taxa (all ascomycetes) possessed one to four unique laccase gene sequences and sequence polymorphisms were not related to fungal taxonomy. Most laccase sequences were species-specific, although two sequences were shared by two or three fungal taxa. Characterizing the

laccase gene is an important step towards measuring enzyme gene expression *in-situ* and resolving the saprotrophic role of ericoid root fungi.

INTRODUCTION

Ericaceous litter polyphenols are hypothesized as an underlying mechanism for soil N retention and seedling suppression by ericaceous plants in many forest ecosystems (Northup et al., 1998; Preston, 1999; Hättenschwiler and Vitousek, 2000; Mallik, 2003). Plant-litter polyphenols can decrease decomposition rates (Basaraba and Starkey, 1966; Benoit et al., 1968) and increase soil N content through the formation of recalcitrant protein-tannin complexes (Handley, 1961). Since polyphenol accumulation in soils can lower N mineralization rates (Bradley et al., 2000; Fierer et al., 2001; Castells et al., 2003; Kraus et al., 2004), litter inputs from polyphenol-rich ericaceous plants may contribute to the suppression of ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) trees. Unlike ECM and AM plants, ericaceous plants themselves are predicted to be less affected by lower availability of inorganic N if they can degrade complex organic substrates and acces N through their saprotrophic ericoid mycorrhizal (ERM) fungi (Read et al., 2004). Since ERM plants tend to grow in nutrient-poor soils, elucidating the structure of ERM fungal communities is valuable step towards understanding the functional attributes these fungi offer their plant hosts (Perotto et al., 2002), including the potential for the degradation of complex organic substrates.

The southern Appalachians support temperate forests with remarkable biological diversity and productivity (Whittaker, 1956; Monk et al., 1985). A common evergreen shrub, *Rhododendron maximum* L., accounts for a significant portion of aboveground biomass in these forests (Monk et al., 1985) and is continuing to increase in coverage (Dobbs and Parker, 2004). *R. maximum* alters patterns in forest community structure by suppressing regeneration and

decreasing the richness of understory plants (Phillips and Murdy, 1985; Nilsen et al., 1999; Beckage et al., 2000; Nilsen et al., 2001; Lambers and Clark, 2003). These shifts in plant community structure are primarily due to a reduction in light under *R. maximum* thickets (Clinton and Vose, 1996; Lambers and Clark, 2003). There is also evidence that *R. maximum* increases organic N storage and decreases inorganic N availability in these soils (Wurzburger and Hendrick, 2007), possibly a result of the quantity and quality of its litter (Hoover and Crossley, 1995; Hunter et al., 2003). Ericoid mycorrhizal fungi of *R. maximum* may allow for the continued acquisition of N from complex organic substrates in these forest soils (Wurzburger et al. Ch. 3), however, the structure and saprotrophic activities of this fungal community are poorly understood.

In previous work, we hypothesized that the impact of *R. maximum* on the N cycle and patterns in N acquisition by mycorrhizal roots are evidence of a plant-soil-mycorrhiza feedback. Protein-tannin complexes derived from *R. maximum* leaf litter tannins are more recalcitrant to decomposition than those from forest trees (Wurzburger, Ch. 3). Furthermore, ERM roots of *R. maximum* acquire more protein-tannin N compared to ECM and AM roots of forest trees and plants (Wurzburger, Ch. 3). Soil microbes and mycorrhizal fungi can contribute to the breakdown of protein-tannin complexes through the production of extracellular enzymes (Bending and Read, 1996a; Bending and Read, 1996b; Bending and Read, 1997). Activities of polyphenol oxidases are elevated in *R. maximum* soils suggesting differences in the structure of soil microbial communities between forests with and without *R. maximum* (Wurzburger and Hendrick, 2007). One anticipated characteristic in the soil microbial community of these forests is the presence of an active ERM fungal community associating with *R. maximum*. However, there is no information on the fungal communities associating with the ERM roots of this shrub,

nor any ericaceous shrub of southern Appalachian forests. Furthermore, we are limited in our ability to directly measure saproptrophic activities of ERM fungi by quantifying gene expression levels because of a paucity of gene sequence data for extracellular enzymes. Laccase is a polyphenol oxidase with ecological importance and has been well studied in basidiomycete decomposer fungi, yet its gene sequence has not been characterized in ERM fungi (Burke and Cairney, 2002).

Although ericaceous plants are common in the understories of temperate and boreal forests world-wide, we are just beginning to document the fungal composition of ERM roots using molecular methods (Perotto et al., 2002; Allen et al., 2003; Vrålstad, 2004). *Oidiodendron maius* and fungi from the *Rhizoscyphus ericae* complex are frequently cultured from ERM roots, yet they are not typically dominants in these communities based upon molecular approaches such as direct amplification and cloning of fungal DNA from ERM roots (Allen et al. 2003; Bougoure and Cairney 2005a). In fact, taxa from the Sebacinaceae were most frequent among cloned fungal DNA from ERM roots of *Gaultheria shallon* (Allen et al., 2003). Furthermore, several unknown or presumably saprotrophic fungal taxa are observed on ERM roots indicating a broader compoisition of fungi associate with these roots than previously believed (Allen et al. 2003; Bougoure and Cairney 2005a; Bougoure and Cairney 2005b).

Our objectives were to characterize the fungal community associated with ERM roots of *R. maximum* using both culture-based and cloning-based techniques. Given the documented richness of plants and ECM fungi in southern Appalachian forests (Hardt and Swank, 1997; Walker and Miller, 2002; Walker et al., 2005), we hypothesized that a rich assemblage of fungi associates with the ERM roots of this shrub. Because of the potential importance of polyphenol oxidases as a means to break down polyphenolic substrates in the soils of these forests, our

second objective was to characterize a portion of the laccase gene sequence in our cultured fungi. We hypothesized that a diversity of laccase gene sequences would be present in these fungi because similar patterns exist with saprotrophic and ECM basidiomycetes (Valderrama et al., 2003; Luis et al., 2004; Luis et al., 2005b) and saprotrophic ascomycetes (Lyons et al., 2003).

MATERIALS AND METHODS

We collected root samples in *R. maximum* thickets of mature hardwood forests with across a high elevation ridge in Coweeta Hydrologic Lab and Nantahala National Forest, NC, USA These forests are composed of northern hardwood species *Quercus rubra* L., *Betula lenta* L. *Betula alleghaniensis* Britt., *Acer rubrum* L., and *Fraxinus americana* L. and soils are Inceptisols. For a more detailed description see Wurzburger and Hendrick (2007).

Culturing fungi from ERM roots

R. maximum ERM hair roots were collected from the O horizon from each of four sampling plots in August 2005 and April 2006 and composited into one sample per plot per sampling date. Hair roots from each composite sample were cleaned of other roots and organic matter under a dissecting microscope, inspected for colonization of fungi and rinsed with sterile water. From each sample, 20 - 30 one cm root sections, for a total of 222 root sections, were surface sterilized following methods of Allen et al. (2003) and plated on potato dextrose agar. Cultures were maintained in the dark at room temperature. Cultures dominated by rapidly growing sporulating fungi were discarded (Bougoure and Cairney 2005), while slow-growing fungi were subcultured from the plated roots. Genomic DNA was extracted from each fungal culture using Qiagen DNeasy Plant Mini Kit (Valencia, CA). The ribosomal DNA (rDNA) internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA gene and ITS2) was amplified from each DNA preparation with fungal specific primers ITS4 and ITS1-F (White et al., 1990; Gardes and Bruns, 1993). ITS

products were typed by restriction fragment length polymorphisms (RFLP) using HinfI and AluI (New England Biolabs, Ipswich, MA)(Wurzburger et al., 2001). The ITS product from each RFLP type was sequenced in both directions.

Cloning fungal ITS products from ERM roots

In August 2005, we randomly sampled three 10 cm deep and 2 cm diameter soil cores from each of the four sampling plots. Soil cores were divided into O and A horizons, and hair roots from each horizon were sorted, cleaned of soil and organic material, and rinsed with sterile DI water. Ten to twenty one cm root fragments were subsampled from each soil horizon root sample. Roots were lyophilized, extracted for DNA and the rDNA ITS region was amplified using the same methods as above. ITS products were visualized on 2% agarose gels, stained with ethidium bromide and visualized under UV light. Products were gel extracted with Column-Pure DNA Gel Recovery kits (Lamda Biotech, St. Louis, MO), and eluted with sterile DI water. Purified products were cloned into the pCR2.1 vector with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). From each sample, we purified plasmid DNA from 20-30 *Escherichia coli* colonies using lithium chloride minipreps (Ausubel et al., 1998). Plasmid inserts were amplified using M13For/Rev primers, and RFLP-typed using HinfI and AluI. We utilized RFLP typing of the ITS region in order to pare down the number of sequence samples within (but not between) each root sample; one clone per RFLP type per core was sequenced. At this stage in the analysis, we considered the number of ITS-RFLP types an overestimate of the number of ITS-types because the anticipated presence of small subunit (SSU) Group I introns (Perotto et al., 2000). Introns were removed from sequences during sequence alignment and analysis. Plasmid DNA from each RFLP-type was purified using Qiagen Plasmid miniprep purification kits (Valencia, CA) and sequenced in both directions.

We inspected sequence chromatograms, trimmed vector sequences and aligned bi-directional sequences using SeqMan software (DNASTAR Madison, WI). SSU introns were removed based upon alignments with known sequences in Genbank. A minimum of 97% identity among ITS sequences was used to define fungal taxa as a conservative estimate of taxonomic richness (O'Brien et al., 2005). A BLAST nucleotide search (Altschul et al., 1997) was conducted on individual sequences to determine their taxonomic affinities. In general, the family, order or class that captured all the top BLAST hits was used as a putative classification for each fungal taxon. Sequences with 99-100% identity across the entire ITS1-5.8s-ITS2 span were classified to the species level. Sequences with a 0 e-value and >95% identity with known sequences were classified to the genus level in taxonomically well resolved families. In several cases, unknown, uncultured fungi dominated the BLAST hits making classification problematic. Taxa were classified to phylum if the 5.8s and at least half of the ITS1 or ITS2 regions provided similarity with known sequences. Chimeric sequences, an artifact of cloning DNA from environmental samples (O'Brien et al., 2005), were identified when the ITS1 and ITS2 regions differed in their taxonomic identity.

As a secondary test of taxonomic identity, sequences of ITS fungal taxa resolved beyond phylum were aligned in ClustalX (Thompson et al., 1997) along with ITS sequences of known taxa from Genbank. These alignments were adjusted by eye in Seaview (Galtier et al., 1996) and used for phylogenetic analyses in PAUP v4.0.b10 (Swofford, 2003) using ITS sequences from the Chytridiomycota as an outgroup. First, we conducted a maximum parsimony analysis with a heuristic search, followed by 500 bootstrap replicates. Second, neighbor-joining distance methods and 1000 bootstrap replicates were used to analyze the sequence alignments. Helotiaceae sequences were subjected to additional phylogenetic analyses using a published ITS

alignment of the former *Hymenoscyphus ericae* (Read) Korf and Kernan. aggregate (Hambleton and Sigler, 2005) to further resolve the identities of these fungal taxa.

Fungal community richness and similarity

We estimated fungal richness in the O and A horizons using EsimateS software based upon the frequency of cloned ITS sequences among sampled soil cores (Colwell, 2005). Because of unequal clone sampling between the O and A horizon, we produced taxa accumulation curves from fungal taxa abundance data from each subsample of roots (1000 bootstrap replicates with replacement using the Chao1 richness estimator; Chao, 2004). These richness estimates were plotted against the number of sampled clones. We used Chao abundance based Sorensen indices to analyze community similarity between soil horizons both within and between cores from each sampling plot (Colwell, 2005).

Laccase gene sequences

The *lcc2* region of the laccase gene (between copper binding domains II and III) was amplified from genomic DNA of each fungal culture using the degenerate primers LAC3FOR and LAC4REV (Lyons et al., 2003). Because laccase sequences are not related to taxonomy (Valderrama et al., 2003), we only screened our cultured fungal collection in order to assign laccase sequence types to an individual fungal taxon. For each 25 µl reaction 50-100 ng of template DNA was combined with 2.5 µl PCR buffer, 20 µM each dNTP, 2 µM each primer, 2.5 mM $MgCl_2$ and 5 U Taq DNA polymerase, under cycling conditions described in Lyons et al. (2003). PCR products were visualized under UV light on 2% agarose gels stained with ethidium bromide. Products of anticipated size (0.9 -1kb) were gel extracted and cloned following the same methods as above. From each reaction, plasmid DNA from 10 *E. coli* colonies was purified, amplified with M13For/Rev primers and digested with restriction enzymes (as above).

One plasmid insert from each restriction fragment type from each sample was sequenced in both directions and compared to Genbank via BLASTx (Altschul et al., 1997). Nucleotide sequences with similarity to known laccase protein sequences (or hypothetical proteins) were retained and bi-direction sequences were aligned. The correct reading frame was determined from translated nucelotide sequences using MapDraw (DNASTAR Madison, WI), and introns were removed following the GT-AG splice junctions of fungi (Bon et al., 2003; Spingola et al., 1999). Amino acid sequences were entered into a BLASTp search in Genbank to determine the closest laccase sequence match. Each laccase nucleotide sequence and 18 additional ascomycetous laccase sequences were aligned in Clustal X (Thompson et al., 1997). The alignment was adjusted by eye in Seaview (Galtier et al., 1996), and used for phylogenetic analyses as above. First, alignments were subjected to a maximum parsimony analysis using a heuristic search and bootstrapping was performed with 100 replications. Second, a neighbor-joining tree was constructed using the Kimura-2 parameter distance option and replicated with 1000 bootstrap replicates. Basidiomyceteous laccase gene sequences were used as an outgroup for all trees.

RESULTS

Cultured fungi and cloned fungal ITS types

ERM roots possess a fine scale of fungal richness because individual root cortical cells can be colonized by individual species of fungi (Perotto et al., 2002; Allen et al., 2003). To capture this potential diversity, we cultured fungi from ERM roots and cloned fungal DNA from subsamples of ERM roots. We isolated 52 cultures of fungi from 222 plated ERM root segments, representing 17 unique ITS fungal taxa (Table 4.1). We successfully amplified fungal ITS products from ERM roots from 11 of 12 soil cores from the four sampling plots. Fungal ITS products were attained from 11 O horizon root subsamples. Nine of the cores possessed ERM

roots in the A horizon, and DNA extracts from five of these amplified successfully. On average, we sampled 25 clones per root subsample from which we obtained an average of 20 fungal ITS products. The remaining clones contained no plasmid insert, an *R. maximum* ITS insert, or provided no sequence data after repeated attempts. From a total of 352 fungal ITS clones we identified 57 unique ITS fungal taxa (Table 4.1). Three cloned fungal ITS types were also observed in the culture collection. In total, we observed 71 fungal ITS types from ERM roots. Four additional ITS types were excluded from the analysis as they were deemed chimeric and 12 sequences with poor matches to those in Genbank (e.g. those assigned to the Kingdom Fungi) could not be reliably screened for chimeras.

Fungal taxonomic identity

Based upon similarities with published sequences in Genbank, the 71 fungal ITS types included members of the Ascomycota (45), Basidiomycota (12), Zygomycota (1), a putative Chytridiomycota (1) and those of unknown classification beyond the Kingdom Fungi (12). Forty-eight taxa were resolved beyond phylum including 36 ascomycetes, 12 basidiomycetes and 1 zygomycete. The taxonomic identities of these 48 fungal ITS types were further supported by phylogenetic analysis with an additional 47 ITS sequences from identified fungi. A maximum parsimony analysis produced 37 trees each with a parsimony score of 5837; a consensus of these was resampled with 100 bootstrap replicates (Figure 4.1). Neighbor-joining methods produced trees of similar structure (not shown). The fungal sequences clustered with their putative identities based upon BLAST searches. Sequences from the three fungal phyla formed three clades, each generating 98, 99 and 90% bootstrap support (Zygomycota, Basidiomycota and Ascomycota, respectively). In the Ascomycota, the Leotiomycetes and Sordariomycetes sequences each clustered with 58% and 98% bootstrap support. The remaining ascomycetes,

clustered together in a weakly supported clade (17%), however, within this, sequences generally clustered by their putative taxonomic identity. To further resolve the identity of 6 fungal taxa with strong BLAST matches to sequences of *Rhizoscyphus ericae* W.Y. Zhuang & Korf, we aligned these sequences with a published alignment of the former *H. ericae* aggregate (Hambleton and Sigler, 2005). Only one fungal taxon from our study, *Rhizoscyphus ericae* c9, aligned with the 3 newly defined clades in the *H. ericae* aggregate. The remaining five were not identified beyond the Helotiaceae.

Relative frequency of fungal taxa

Cultured fungi were dominated by *Oidiodendron maius* Barron, which accounted for 40% of the culture collection and several fungal taxa were cultured only once (Figure 4.2a). A frequency distribution characterized the cloned ITS types, with the most frequent fungal ITS type accounting for 13% of the sampled clones (Figure 4.2b). The 7 most frequent fungal taxa each accounted for > 4% of clones included taxa in the Chaetothyriales, Helotiales, Sebacinales, Sclerodermataceae and an unknown taxon of the Ascomycota. In both the culture and clone fungal collections, singletons (ITS types observed once) accounted for ~50% of the fungal taxa (Figure 4.2).

Richness of ERM root fungi by soil horizon

We observed greater fungal richness on ERM roots in the O horizon (48) compared to the A horizon (23). Since fungal ITS clones were not evenly sampled between the horizons (228 O horizon vs. 134 in A horizon), we further explored fungal richness by generating taxa richness curves by plotting estimated taxa richness against the number of sampled ITS fungal clones (Figure 4.3). The lack of overlap among O and A horizon richness curves and standard error bars suggests a difference in richness accumulation per sampled clone between the two soil horizons.

The comparison of community composition between O and A horizon was complicated by the abundance of singleton taxa. Over half of the fungal taxa were isolated only once, and nearly 70% of taxa were each observed only once among the 11 cores (data not shown). A high percentage of taxa observed in the A horizon only (78%) were unknown fungi or classified only to a phylum, compared to 35% in O horizon-only taxa. Sorensen similarity values were greater between O and A horizon samples from the same soil core (0.34(0.12), mean (SE)) than from samples from different cores within the same sampling plot (0.17(0.06). Therefore, despite differences in the composition of root fungi from the O and A horizon, fungi observed from each horizon from the same core had greater similarity than fungi observed between neighboring cores.

Fungal laccase sequences

We obtained PCR products from the *lcc2* region of the laccase gene from all 17 fungal taxa of the culture collection. Each fungal taxon contained one to four laccase sequence types from a screening of 10 clones from each, totaling 30 unique laccase gene sequences (Table 4.2). Laccase gene sequences were diverse and possessed strong amino-acid sequence similarities (49-98%) with published laccase sequences of ascomycetes. Two fungal taxa (*Dermea* c14 and Sordariales c61) possessed nearly identical laccase gene sequences (98% nucleotide similarity). The Chaetosphaeriaceae c60 type 1 laccase sequence was 98% similar to a previously published laccase sequence from a saprotrophic ascomycete (SAP142) (Lyons et al., 2003). Neighbor-joining analysis indicated that the clustering of laccase gene types was not related to fungal taxonomy and the distribution of laccase gene sequences fell into two clades with 74 and 100% bootstrap support (Figure 4.4).

DISCUSSION

A diversity of ERM root fungi

Despite the world-wide distribution of ericaceous plants and their abundance in the understories of temperate and boreal forests, we know relatively little about the composition and structure of ERM fungal communities (Perotto et al., 2002; Vrålstad, 2004). We observed 71 putative fungal taxa associating with ERM roots of *R. maximum*. These fungal taxa represent four fungal phyla, although a majority of these taxa belong to the Ascomycota. Few studies have utilized direct molecular methods to identify the fungal assemblage of ERM roots (e.g. cloning, density gradient gel electrophoresis) (Berch et al., 2002; Allen et al., 2003; Bougoure and Cairney, 2005a; Bougoure and Cairney, 2005b; Setaro et al., 2006a) and these studies, as well as ours, capture a more diverse assemblage of fungi than that previously observed in culture collections. In our study, 30% of the fungal taxa observed on ERM roots could not be classified beyond the Kingdom Fungi or a fungal phylum using previously published ITS sequences in Genbank. Many root fungi have remained undetected until the use of direct molecular techniques (Vandenkoornenhuyse et al., 2002; Allen et al., 2003). The lack of similarity in the fungal assemblages observed by culturing and molecular methods is not surprising given the biases involved in both culturing and PCR-based identification techniques.

ERM symbionts and potential ERM symbionts

The ecological classification of the ERM root fungi observed in this study remains unclear. The entire assemblage of fungi observed in our study is not likely ERM (e.g. forming hyphal coils in hair roots and providing benefit to the plant host), yet all fungi were either cloned or cultured from ERM hair roots that were cleaned of organic debris and rinsed in sterile H_2O or surface sterilized, respectively. Resynthesis trials would be necessary to determine the ability of these

fungi to form mycorrhizal structures and provide benefit to the host, however in this study a majority of observed fungal taxa were not cultured. We observed two fungal taxa previously documented as ERM symbionts, *R. ericae* and *O. maius* (Xiao and Berch, 1995; Read, 1996). *O. maius* dominated our culture collection, although both taxa each accounted for less than 4% of sampled fungal ITS clones We also observed several taxa that are potential ERM symbionts based upon previous studies including a *Cryptosporiopsis* taxon and several taxa in the Dermataceae, Helotiales and Chaetothryiales (Vrålstad et al., 2002; Allen et al., 2003; Sigler et al., 2005). Two species of *Cryptosporiopsis* are commonly found on roots of oak and ericaceous plants in Europe (Kowalski and Bartnik, 1995; Kowalski et al., 1998; Verkley et al., 2003), but appear infrequently on ERM roots of North American ericaceous plants (Allen et al., 2003; Sigler et al., 2005). In our study, a *Cryptosporiopsis* taxon was observed only among our cultured fungi, representing 10% of the culture collection. In contrast, the four putative taxa in the Chaetothyriales were only observed in our clone collection, and together, represent about 15% of screened ITS fungal clones in our study. Fungi in the Chaetothryiales were observed from ERM roots of *Gaultheria shallon* Pursh in western Canada (Allen et al., 2003), *Erica arborea* L. and *Quercus ilex* L. in Italy (Bergero et al., 2000; Bergero et al., 2003), and the Australian epacrids *Epacris pulchella* Cav. (Bourgoure and Cairney, 2005a) and *Woolsia pungens* Cav. (Muell.) (Midgley et al., 2003). Fungi in the Chaetothryiales can colonize ERM root cortical cells in resynthesis trials, although their effect on plant nutrition and growth is not clear (Bergero et al., 2000; Allen et al., 2003).

Nearly a quarter of the observed fungal taxa in our study belong to the Leotiomycetes; however, only one taxon had a strong affinity to *R. ericae* and the former *H . ericae* aggregate (Hambleton and Sigler, 2005). Fungi of the Leotiomycetes and the Helotiales, in particular, have

a broad ecological range including saprotrophs, parasites, endophytes and ECM and ERM symbionts (Vrålstad et al., 2000; Villarreal-Ruiz et al., 2004; Wang et al., 2006). Some of these fungi may operate between multiple lifestyles (e.g. endophyte, saprotroph) depending on resource availability (Wang et al., 2006). Our perceptions of fungal lifestyle and ecology are based upon the environment in which we observe fungi, and our ability to now directly detect fungi in roots using molecular techniques may reveal broader lifestyle patterns in the Helotiales than previous believed (Abeln et al. 2000; Wang et al., 2006).

Fungal taxa from the Sebacinales were cloned from ERM roots of *R. maximum* in our study and from ERM roots of *G. shallon* in a previous study (Allen et al., 2003). The newly defined Sebacinales (heterobasidiomycetes) contains a diversity of fungi including ECM, ERM, orchid mycorrhizas, and newly described jungermannioid and cavendishioid mycorrhizas (Weiss et al., 2004; Setaro et al., 2006a; Setaro et al., 2006b). Based upon similarities with sebacinalean sequences described in Weiss et al. (2004), the four taxa in our study likely fall into subgroup B, which may represent a species complex, closely related to *Sebacina vermifera* Warcup & Talbot (Weiss et al., 2004; Setaro et al., 2006a). Before the use of direct molecular detection, basidiomycetes were observed on roots of ericaceous plants based upon anatomical details although their identity was unknown (Bonfante-Fasalo et al., 1980; Peterson et al., 1980). Based upon ultrastructural observations of South American ericaceous plant roots, sebacinalean fungi can also co-colonize individual cortical cells of ERM roots with ascomycetous fungi (Setaro et al., 2004b). The ecological role that sebacinalean fungi play in ERM roots is not fully understood, however, they are clearly common associates of ERM roots, as they accounted for 16% of screened fungal ITS clones from *R. maximum* ERM roots in our study and nearly 60% of clones from *G. shallon* ERM roots in a previous study (Allen et al., 2003).

Ectomycorrhizal and saprotrophic fungi observed on ERM roots

We also observed several ECM fungi associating with *R. maximum* ERM roots in our clone collection including *Cenococcum geophilum* Fr. and taxa from the genera *Elaphomyces*, *Russula*, *Lactarius*, *Tomentella* and families Russulaceae, Tricholomataceae and Sclerodermataceae. Although most of these taxa were singletons, a Sclerodermataceae taxon was relatively frequent and sampled from 16 clones between two soil cores. The colonization of ERM roots by ECM fungi (Smith et al., 1995) and the appearance of fungal mantles on ERM roots (Dighton and Coleman, 1992; Wurzburger and Bledsoe, 2001) have been observed in the past, as has simultaneous colonization of ERM and ECM roots by a single mycelium of *Cadophora finlandia* (Wang & Wilcox) Harrington & McNew (Villareal-Ruiz et al., 2004). The potential for ECM fungi to colonize ERM roots seems considerable since ERM plants typically grow in forests dominated by ECM hosts, and roots of these plants intermingle in soils. However, it is unknown if the observation of ECM fungi on ERM roots in our study indicates either substantial root colonization or a benefit to the host plant.

Several fungal taxa previously described as soil or litter saprotrophs were also observed on ERM roots, including the commonly observed soil fungi *Verticillium bulbillosum* W. Gams & Malla, *Trichoderma koningii* Oudem. and *Mortierella humilis* Linnem. ex W. Gams. (Dix and Webster, 1995). We also observed two newly described species, *Ophiostoma dentifundum* Aghayeva & M.J. Wingf., and *Fulvoflamma eucalypti* Crous., which have been isolated from oak wood and eucalyptus leaf-litter, respectively (Aghayeva et al., 2005; Crous et al., 2006). In addition, we observed a cosmopolitan plant pathogen, *Glomerella acutata* J.C.Guerber & J.C.Correll, which infects species of *Rhododendron* and is responsible for leaf anthracnose and root rot (Sreenivasaprasad and Talhinhas, 2005). We observed a number of putative saprotrophic

taxa in the genera *Xylaria*, *Hypoxylon*, *Chaetosphaeria*, *Pestalotiopsis*, *Pilidium* and unknown taxa in the Leotiomycetes Dothideomycetes, Sordariomycetes and Aphyllophorales (Dix and Webster, 1995). The abundance of these apparently non-mycorrhizal fungi suggests that we detected soil saprotrophic fungi despite our attempts to remove them from the rhizosphere of sampled roots. Another possibility is that ERM fungi loosely associate with a diversity of fungi including saprotrophs and endophytes (Perotto et al., 2002). If some of these taxa are root endophytes they can still provide benefit to the plant host, albeit indirectly (Jumpponen and Trappe, 1998). The low specificity of root associated fungi may allow ericaceous plants to indirectly benefit from the saprotrophic activities of these fungi (e.g. extracellular enzyme production), particularly in organic, nutrient-poor soils.

ERM root fungal richness greater in organic soils

Fungal richness was greater on ERM roots from the O horizon than from the A horizon in total observed taxa and estimated richness measures. Fungal richness decreased from organic to mineral horizons in forest soils (O'Brien et al., 2005), and perhaps the richness of ERM root fungi follows a similar pattern. Analyzing the fungal community composition of each soil horizon is difficult since nearly 70% of the fungal taxa in this study were each observed once from the 11 soil cores, and 50% of the taxa were sampled once from the 352 clones. However, the percentage of unknown fungi and fungi not classified beyond a phylum was greater among the taxa only observed in the A horizon compared to those only observed in the O horizon. This pattern in our study, as well as the frequency of unknown fungi from forest soils, suggests we know less about soil and root fungi from mineral compared to organic soil horizon (O'Brien et al., 2005).

Laccase gene sequences are polymorphic in ERM root fungi

The second objective of our research was to characterize a portion of the laccase gene sequence in ERM fungi. Laccase is a polyphenol oxidase enzyme with low substrate specificity and considerable potential to degrade recalcitrant organic substrates (Burke and Cairney, 2002; Leonowicz et al., 2001) and polymerize toxic phenolics (Bending and Read, 1997). Laccase production by ERM fungi may allow for greater access to N within complex organic substrates for ERM plants. However, laccase gene sequences have not been documented in ERM fungi. The laccase gene has been characterized in many saprotrophic and ECM fungi, (Burke and Cairney, 2002; Chen et al., 2003; González et al., 2003; Lyons et al., 2003; Valderrama et al., 2003; Luis et al., 2004; Luis et al., 2005a; Luis et al., 2005b) but these have been predominantly basidiomycetes.

From amplified portions of the laccase gene (*lcc2* region) we observed at least one, and as many as four, unique laccase sequence types per fungal taxon of our culture collection. Multiple copies of the laccase gene are common among fungi (Valderrama et al., 2003; Hoegger et al., 2006) and in previously studied saprotrophic basidiomycetes, individual species possessed as many as five laccase isozymes (e.g. *Trametes* spp., Hoshida et al., 2002). Copies of laccase gene sequences represent silent, inducible or constitutively expressed genes (Valderrama et al., 2003). Multiple copies of laccase isozymes appear to be more common among saprotrophic compared to ECM basidiomycetes (Luis et al., 2004). The *lcc2* region, the focus of our study, may also be more polymorphic among fungi and gene sequences compared to the better studied, yet shorter segment of the *lcc1* region (Luis et al., 2004; Luis et al., 2005; Pointing et al., 2005).

Although fungi possess several laccase isozymes and their nucleotide sequences are highly polymorphic, laccase gene sequences in the Basidiomycota are species-specific

(Valderrama et al., 2003; Luis et al., 2004). Gene multiplicity and species-specificity suggests that laccase gene diversity may be the result of independent divergence events within basidiomyceteous taxa (Valderrama et al., 2003). Species-specificity of laccase gene sequences was generally true among our 17 ascomycetes, with two exceptions. One nearly identical (98% nucleotide identity) laccase gene sequence was observed in two unrelated fungal taxa (*Dermea* c14 and Sordariales c61). Also, a laccase gene sequence from the taxon Chaetosphaeriaceae c60 (type 1) in our study possessed a 98% identity with a laccase nucleotide sequence observed in two different saprotrophic ascomycetes from a previous study (*Lachnum* sp. and SAP142, Lyons et al., 2003). These observations suggest that laccase gene sequences may not be entirely species-specific in the Ascomycota, revealing a potentially different evolutionary pathway of the gene. Our screening of 10 clones per fungal taxon was not exhaustive, but revealed the potential for diverse laccase gene sequences among ERM root fungi.

Conclusions

A diversity of fungi associating with plant roots is not uncommon. However, the 71 fungal taxa observed on ERM roots in this study present a number of unanswered questions about the ecology of this fungal community. To date, only two true ERM symbionts (or species complexes) have been verified (*R. ericae* and *O. maius*), both of which were observed in this study. We also observed a number of possible ERM symbionts, many of which belong to the Helotiaceae, as well as the Chaethyriales and Sebacinales. Together, these known symbionts and possible symbionts account for 30% of the observed taxa and 53% of the observed ITS fungal clones from ERM roots of *R. maximum* in our study. Clearly we are lacking an understanding of the symbiotic and endophytic potential of these diverse root fungi. More likely, a distinct

classification between root endophytes and mutualists is not realistic, and we need to consider root-fungal relationships operating with a diversity of functions (Egger, 2006).

ERM root fungi are valuable saprotrophs and contribute to decomposition and plant nutrient acquisition in organic soils. Extracellular enzyme production is important in this context, yet we know little about enzyme production and expression *in situ*. We observed a diversity of laccase gene sequences in ascomyceteous ERM root fungi, most of which are species-specific. The characterization of laccase gene sequences in ERM root and soil fungi is valuable for future research. These laccase gene sequences can aid in the development of methods to target species-specific laccase mRNA and measuring expression levels in soils to further our understanding of the saprotrophic role of ERM fungi.

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Table 4.1 Putative identity and closest BLAST match of fungal taxa observed from ERM roots of *Rhododendron maximum*.

Fungal phylum and class taxonomic identity	Closest BLAST match	E-value	Identity (%)
Ascomycota			
Chaetothyriomycetes			
Chaetothyriales c2	*Capronia villosa* (AF050261)	3 x e^{-101}	91
Chaetothyriales c6	*Capronia villosa* (AF050261)	5 x e^{-100}	98
Chaetothyriales c42	*Capronia villosa* (AF050261)	2 x e^{-90}	96
Chaetothyriales c50	*Capronia villosa* (AF050261)	3 x e^{-98}	98
Dothideomycetes			
Cenococcum geophilum c44	*Cenococcum geophilum* (AY394919)	0	97
Dothideomycetes c87	*Massarina corticola* (AF383957)	6 x e^{-124}	94
Eurotiomycetes			
Elaphomyces c52	*Elaphomyces muricatus* (DQ974740)	0	94
Oidiodendron maius c32	*Oidiodendron maius* (AF062800)	0	99
Sordariomycetes			
Sordariomycetes c58	*Cephalotheca sulfurea* (AB278194)	9 x e^{-114}	89
Verticillium bulbillosum c24	*Verticillium bulbillosum* (AJ292410)	0	100
Trichoderma koningii c35	*Hypocrea koningii* (AJ301990)	0	99
Sordariales c61	*Chaetomium globosum* (DQ854987)	4 x e^{-171}	98
Chaetosphaeria c48	*Chaetosphaeria chloroconia* (AF178542)	0	96
Chaetosphaeriaceae c60	*Chaetosphaeria chloroconia* (AF178542)	0	94
Xylaria c63	*Xylaria arbuscula* (AYI83369)	0	94
Hypoxylon c57	*Hypoxylon perforatum* (AJ390407)	0	99
Pestalotiopsis c84	*Pestalotiopsis maculans* (AF405296)	0	96
Glomerella acutata c85	*Glomerella acutata* (DQ286128)	0	100
Ophiostoma dentifundum c88	*Ophiostoma denifundum* (AY495435)	0	100
Leotiomycetes			
Leotiomycetes c17	*Phialophora finlandia* (AF486119)	1 x e^{-143}	90
Helotiaceae c1	*Rhizoscyphus ericae* (AY394907)	0	94
Helotiaceae c7	*Rhizoscyphus ericae* (AY394907)	0	93
Rhizoscyphus ericae c9	*Rhizoscyphus ericae* (AY394907)	0	98
Helotiaceae c10	*Rhizoscyphus ericae* (AY394907)	0	93
Helotiaceae c26	*Rhizoscyphus ericae* (AY394907)	4 x e^{-136}	93
Helotiaceae c40	*Rhizoscyphus ericae* (AY394907)	7 x e^{-173}	93
Dermea c14	*Dermea viburni* (AF141163)	0	96
Cryptosporiopsis c80	*Cryptosporiopsis melanigena* (AF141196)	0	96
Dermataceae c79	*Cryptosporiopsis actinidae* (AY359234)	0	91
Helotiales c19	*Arachnopeziza aurata* (AAU57496)	0	95
Helotiales c23	*Hyphohdiscus hymeniophilus* (DQ227264)	2 x e^{-179}	93
Helotiales c59	*Arachnopeziza aurata* (AAU57496)	0	92
Helotiales c74	*Phialocephala dimorphospora* (AY606302)	2 x e^{-107}	94
Helotiales c82	*Cadophora finlandica* (DQ485204)	0	95
Pilidium c86	*Pilidium acerinum* (AY487091)	0	97
Fulvoflamma eucalypti c83	*Fulvoflamma eucalypti* (DQ195779)	0	100
Ascomycota c12	*Alatospora acuminata* (AY204589)	6 x e^{-111}	92
Ascomycota c15	*Leptodontidium elatius* (AY781230)	1 x e^{-169}	90
Ascomycota c16	*Alatospora acuminata* (AY204589)	2 x e^{-111}	91
Ascomycota c18	*Alatospora acuminata* (AY204590)	6 x e^{-152}	92

Ascomycota c27	*Mycosphaerella suberosa (AY626985)*	4 x e^{-128}	91
Ascomycota c30	*Berkelella stilbigera* (AB208109)	9 x e^{-102}	98
Ascomycota c38	*Trimmatostroma cordae (AJ244263)*	9 x e^{-99}	93
Ascomycota c41	uncultured ascomycota (AY969712)	6 x e^{-115}	96
Ascomycota c81	*Gyoerffyella rotula* (AY729937)	0	97
Basidiomycota			
Heterobasidiomycetes			
Sebacinales c13	Salal root associated fungus (AF300772)	4 x e^{-134}	95
Sebacinales c21	Salal root associated fungus (AF284137)	4 x e^{-175}	94
Sebacinales c25	Salal root associated fungus (AF284137)	2 x e^{-173}	94
Sebacinales c34	Salal root associated fungus (AF300772)	4 x e^{-147}	97
Homobasidiomycetes			
Tomentella c73	*Tomentella sublilicina* (AF272929)	0	97
Russulaceae c49	*Lactarius volemus* (AY606959)	3 x e^{-145}	92
Russula c56	*Russula raoultii* (AY061712)	0	96
Lactarius c55	*Lactarius camphoratus* (AJ889960)	0	96
Tricholomataceae c46	*Mycena galopus* (AY805614)	0	93
Boletales c72	*Scleroderma bovitsa* (AB211267)	7 x e^{-170}	89
Sclerodermataceae c51	*Scleroderma bovitsa* (AB211267)	0	90
Aphyllophorales c31	*Trechispora hymenocystis* (AF347090)	0	97
Zygomycota			
Mortierella humilis c54	*Mortierella humilis* (AJ878778)	0	99
Chytridiomycota			
Chytridiomycota c77	*Podochytrium dentatum* (DQ536501)	4 x e^{-110}	98
Unknown Fungi			
Fungi c11	*Leptodontidium elatius* (AY781230)	4 x e^{-89}	97
Fungi c28	Fungal endophyte (AY700138)	3 x e^{-92}	98
Fungi c29	*Marchandiomyces aurantiacus* (AY583324)	8 x e^{-86}	98
Fungi c62	*Nectria plagianthi* (AF178417)	3 x e^{-70}	93
Fungi c65	uncultured fungus (AM2060816)	2 x e^{-59}	93
Fungi c66	uncultured fungus (DQ309164)	2 x e^{-130}	88
Fungi c67	ECM root tip (AF4769851)	0	96
Fungi c68	uncultured fungus (AM260905)	0	91
Fungi c69	uncultured fungus (AM260905)	3 x e^{-141}	90
Fungi c70	uncultured fungus (DQ309136)	1 x e^{-33}	93
Fungi c71	uncultured fungus (AM260932)	8 x e^{-32}	100
Fungi c75	Chytridiales sp. JEL187 (AY997935)	9 x e^{-75}	95

Figure 4.1 Maximum parsimony tree (heuristic search) from an ITS sequence alignment with 48 fungal sequences observed from ERM roots of *Rhododendron maximum* and 47 additional sequences of known fungi from Genbank. A chytridiomycete ITS sequence provided an outgroup. Values are percentages from 100 bootstrap replicates.

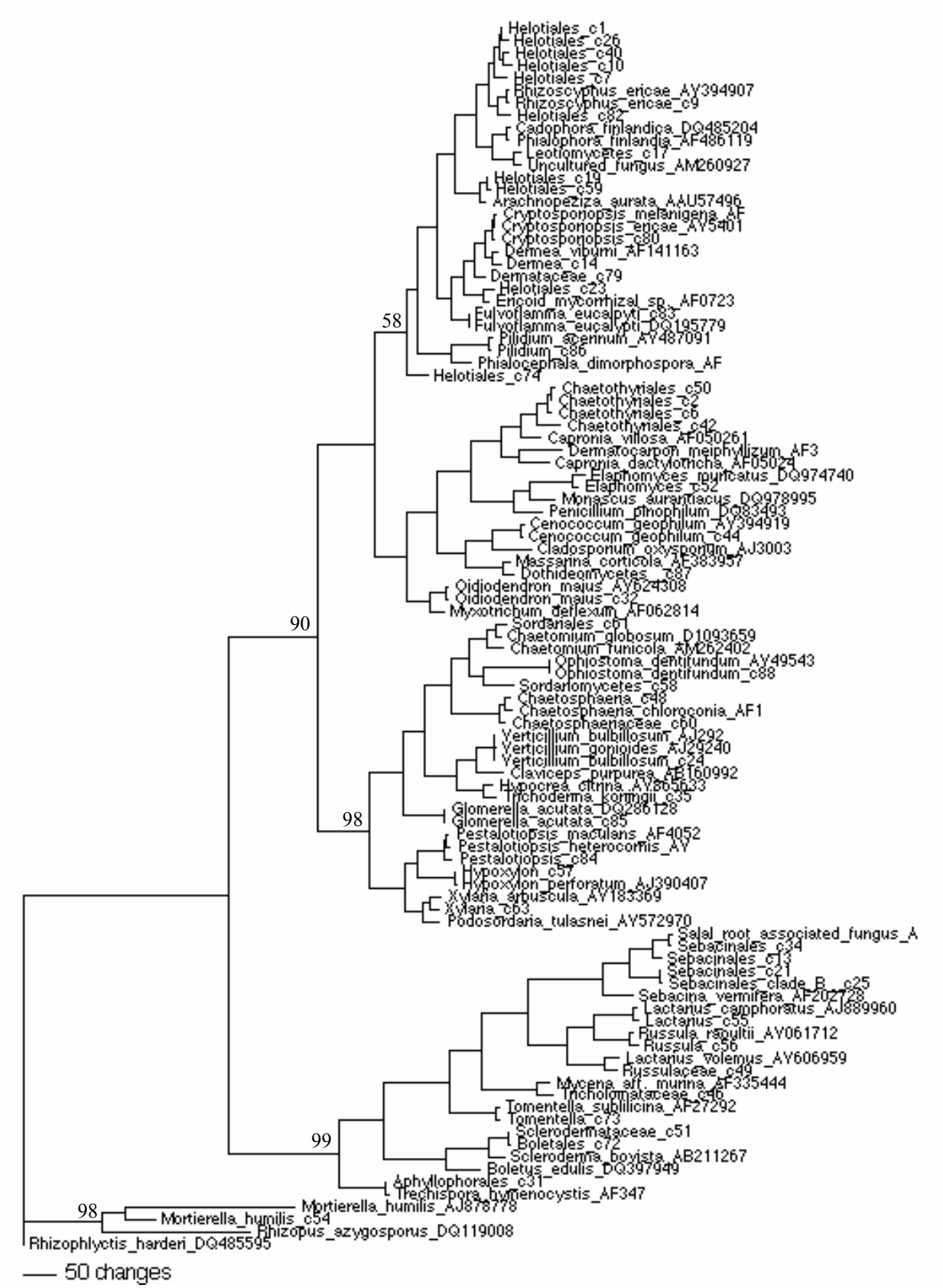

Helotiales_c1
Helotiales_c26
Helotiales_c40
Helotiales_c10
Helotiales_c7
Rhizoscyphus_ericae_AY394907
Rhizoscyphus_ericae_c9
Helotiales_c82
Cadophora_finlandica_DQ485204
Phialophora_finlandia_AF486119
Leotiomycetes_c17
Uncultured_fungus_AM260927
Helotiales_c19
Helotiales_c59
Arachnopeziza_aurata_AAU57496
Cryptosporiopsis_melanigena_AF
Cryptosporiopsis_ericae_AY5401
Cryptosporiopsis_c80
Dermea_viburni_AF141163
Dermea_c14
Dermateaceae_c79
Helotiales_c23
Ericoid_mycorrhizal_sp._AF0723
Fulvoflamma_eucalypti_c83
Fulvoflamma_eucalypti_DQ195779
Pilidium_acerinum_AY487091
Pilidium_c86
Phialocephala_dimorphospora_AF
Helotiales_c74
58
Chaetothyriales_c50
Chaetothyriales_c2
Chaetothyriales_c6
Chaetothyriales_c42
Capronia_villosa_AF050261
Dermatocarpon_meiophyllizum_AF3
Capronia_dactylotricha_AF05024
Elaphomyces_puncatus_DQ974740
Elaphomyces_c52
Monascus_aurantiacus_DQ978995
Penicillium_pinophilum_DQ83493
Cenococcum_geophilum_AY394919
Cenococcum_geophilum_c44
Cladosporium_oxysporum_AJ3003
Massarina_corticola_AF383957
Dothideomycetes_c87
Oidiodendron_maius_AY624308
Oidiodendron_maius_c32
Myxotrichum_deflexum_AF062814
90
Sordariales_c61
Chaetomium_globosum_D1093659
Chaetomium_funicola_AM262402
Ophiostoma_dentifundum_AY49543
Ophiostoma_dentifundum_c88
Sordariomycetes_c58
Chaetosphaeria_c48
Chaetosphaeria_chloroconia_AF1
Chaetosphaeriaceae_c60
Verticillium_bulbillosum_AJ292
Verticillium_goniodes_AJ29240
Verticillium_bulbillosum_c24
Claviceps_purpurea_AB160992
Hypocrea_citrina_AY865633
Trichoderma_koningii_c35
Glomerella_acutata_DQ286128
98
Glomerella_acutata_c85
Pestalotiopsis_maculans_AF4052
Pestalotiopsis_heterocornis_AY
Pestalotiopsis_c84
Hypoxylon_c57
Hypoxylon_perforatum_AJ390407
Xylaria_arbuscula_AY183369
Xylaria_c63
Podosordaria_tulasnei_AY572970
Salal_root_associated_fungus_A
Sebacinales_c34
Sebacinales_c13
Sebacinales_c21
Sebacinales_clade_B_c25
Sebacina_vermifera_AF202728
Lactarius_camphoratus_AJ889960
Lactarius_c55
Russula_rosultii_AY061712
Russula_c56
Lactarius_volemus_AY606959
Russulaceae_c49
Mycena_aff._murina_AF335444
Tricholomataceae_c46
Tomentella_sublilicina_AF27292
Tomentella_c73
99
Sclerodermataceae_c51
Boletales_c72
Sclerodermа_bovista_AB211267
Boletus_edulis_DQ397949
Aphyllophorales_c31
Trechispora_hymenocystis_AF347
98
Mortierella_humilis_AJ878778
Mortierella_humilis_c54
Rhizopus_azygosporus_DQ119008
Rhizophlyctis_harderi_DQ485595
50 changes

Figure 4.2 Relative frequency of fungal taxa observed from ERM roots of *Rhododendron maximum* from a) cultured fungi (52 fungal cultures) from the O horizon, and b) cloned fungal ITS sequences (352 sampled clones) from the O and A horizons.

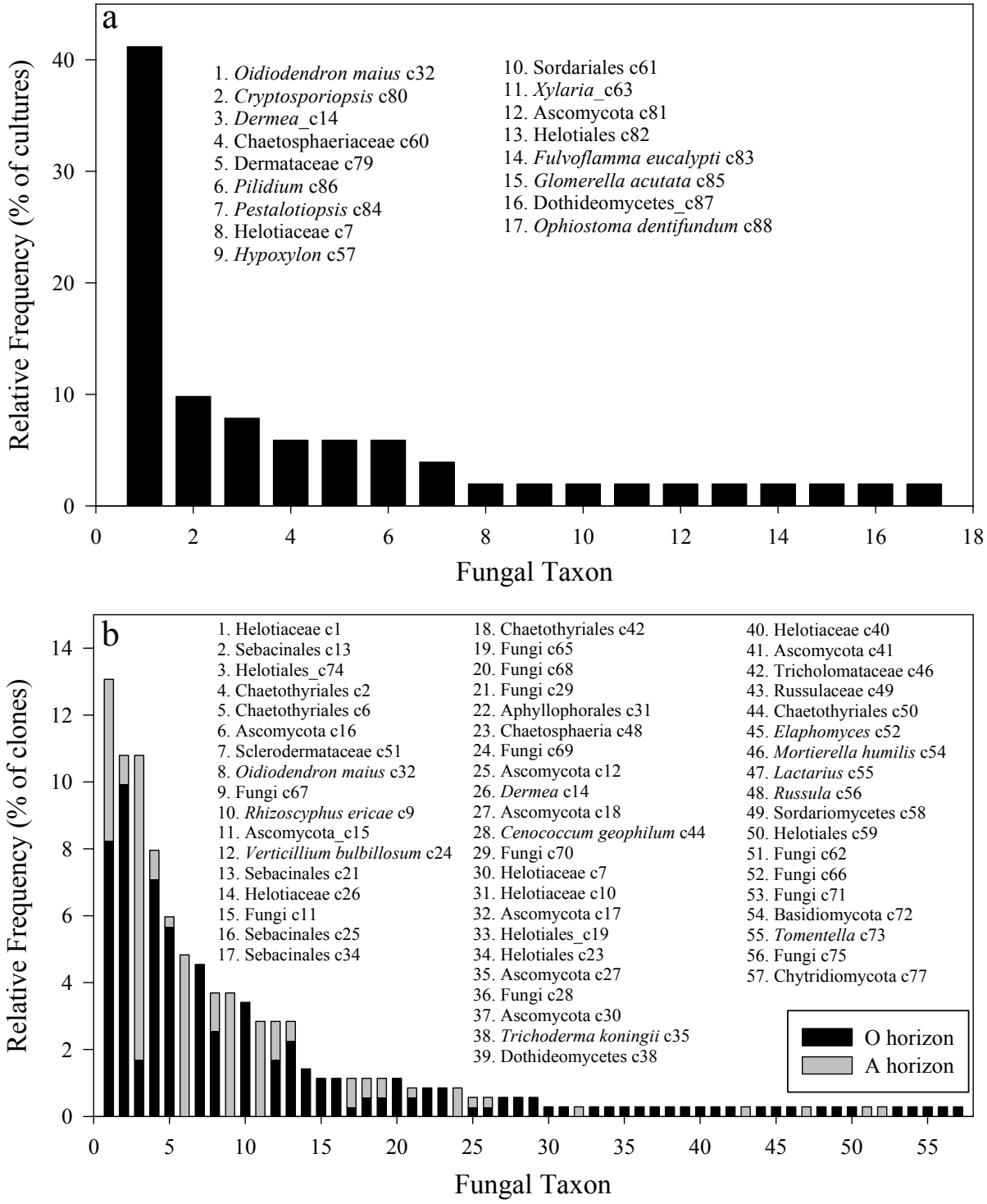

a
Relative Frequency (% of cultures)
Fungal Taxon
1. Oidiodendron maius c32
2. Cryptosporiopsis c80
3. Dermea_c14
4. Chaetosphaeriaceae c60
5. Dermataceae c79
6. Pilidium c86
7. Pestalotiopsis c84
8. Helotiaceae c7
9. Hypoxylon c57
10. Sordariales c61
11. Xylaria_c63
12. Ascomycota c81
13. Helotiales c82
14. Fulvoflamma eucalypti c83
15. Glomerella acutata c85
16. Dothideomycetes_c87
17. Ophiostoma dentifundum c88
b
Relative Frequency (% of clones)
Fungal Taxon
1. Helotiaceae c1
2. Sebacinales c13
3. Helotiales_c74
4. Chaetothyriales c2
5. Chaetothyriales c6
6. Ascomycota c16
7. Sclerodermataceae c51
8. Oidiodendron maius c32
9. Fungi c67
10. Rhizoscyphus ericae c9
11. Ascomycota_c15
12. Verticillium bulbillosum c24
13. Sebacinales c21
14. Helotiaceae c26
15. Fungi c11
16. Sebacinales c25
17. Sebacinales c34
18. Chaetothyriales c42
19. Fungi c65
20. Fungi c68
21. Fungi c29
22. Aphyllophorales c31
23. Chaetosphaeria c48
24. Fungi c69
25. Ascomycota c12
26. Dermea c14
27. Ascomycota c18
28. Cenococcum geophilum c44
29. Fungi c70
30. Helotiaceae c7
31. Helotiaceae c10
32. Ascomycota c17
33. Helotiales_c19
34. Helotiales c23
35. Ascomycota c27
36. Fungi c28
37. Ascomycota c30
38. Trichoderma koningii c35
39. Dothideomycetes c38
40. Helotiaceae c40
41. Ascomycota c41
42. Tricholomataceae c46
43. Russulaceae c49
44. Chaetothyriales c50
45. Elaphomyces c52
46. Mortierella humilis c54
47. Lactarius c55
48. Russula c56
49. Sordariomycetes c58
50. Helotiales c59
51. Fungi c62
52. Fungi c66
53. Fungi c71
54. Basidiomycota c72
55. Tomentella c73
56. Fungi c75
57. Chytridiomycota c77
O horizon
A horizon

Figure 4.3 Species-effort curves generated from estimations of fungal taxa richness on ERM roots of *Rhododendron maximum* from O and A soil horizons. Chao1 estimates of richness (± analytical standard errors) generated from 1000 bootstrap replicates.

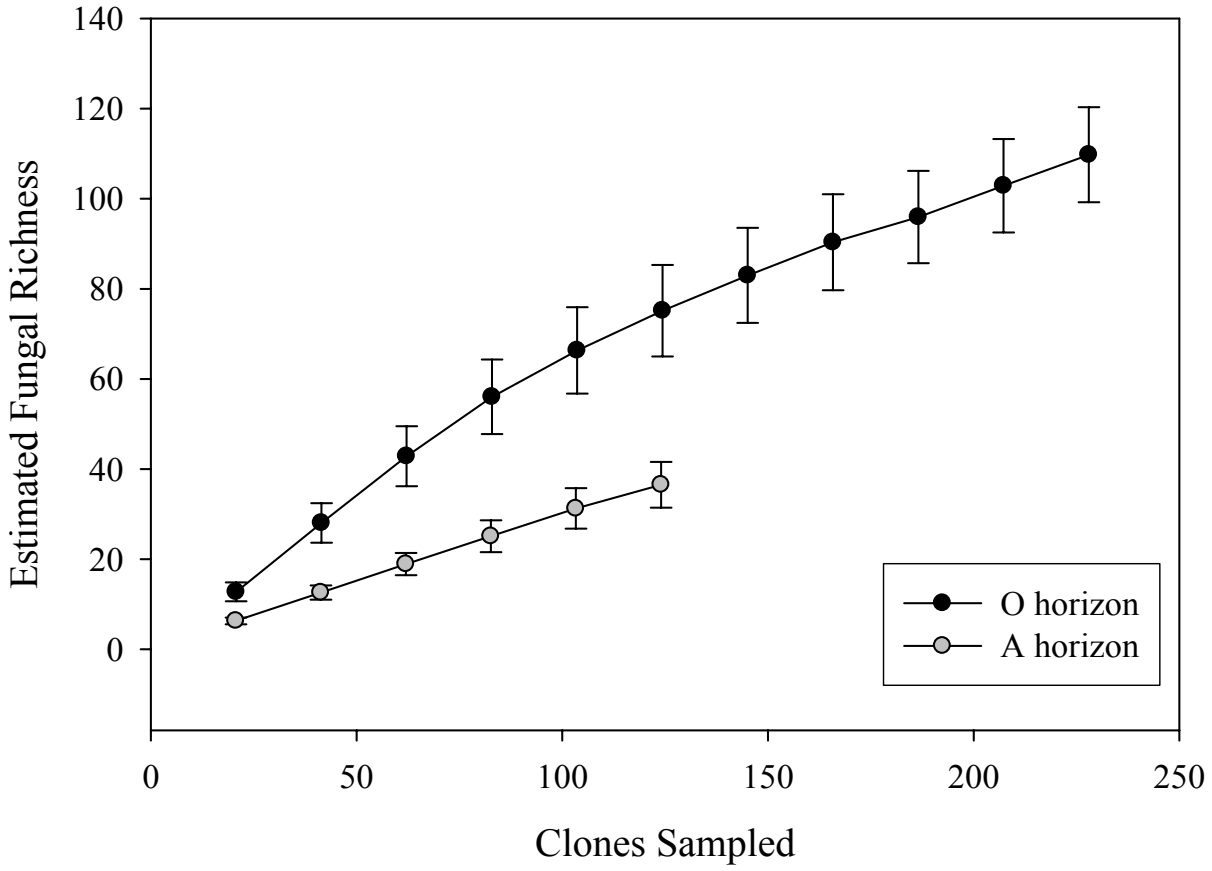
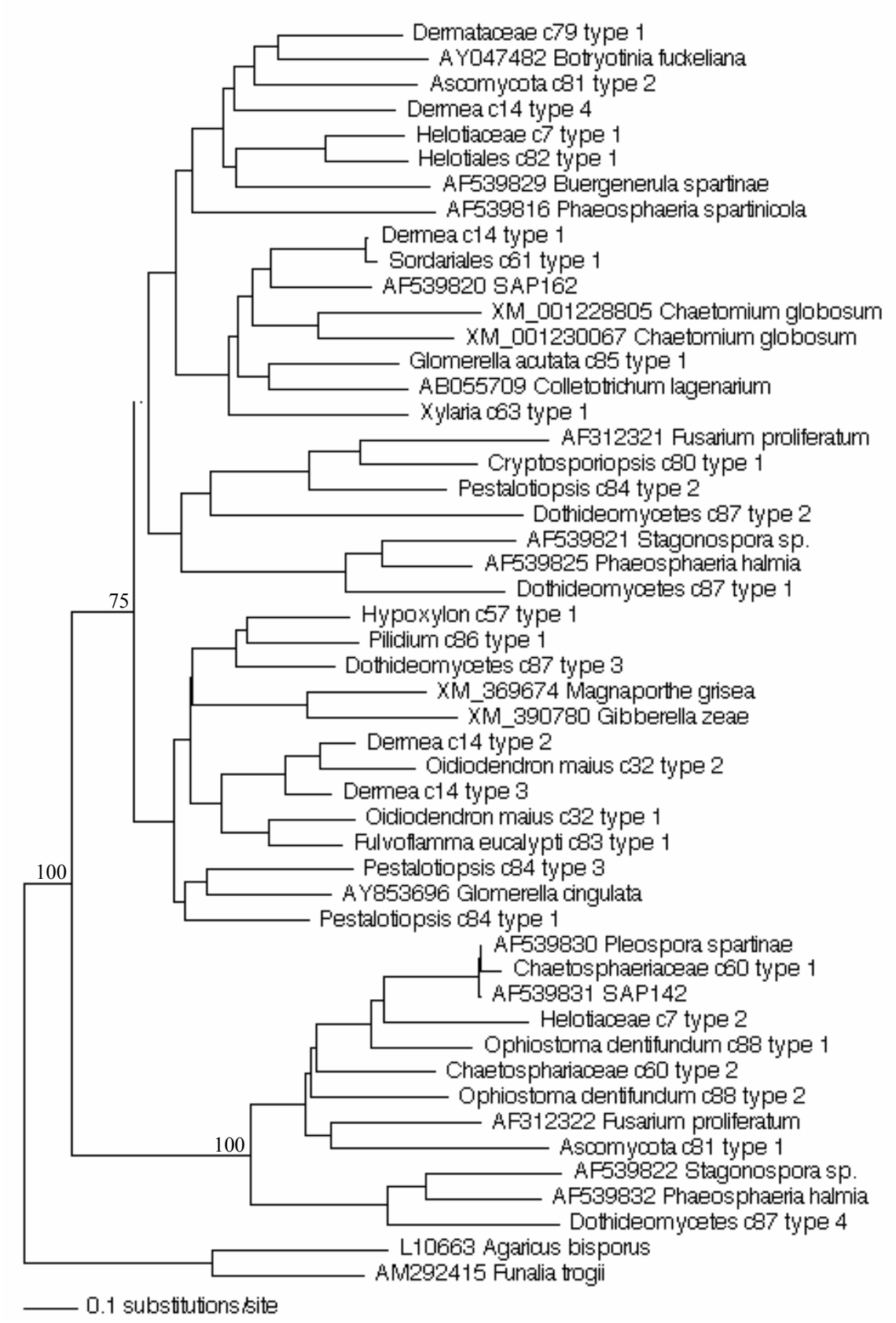


Table 4.2 Fungal laccase gene sequence types and closest BLASTp match from sequences in Genbank.

Fungal taxonomic identity and laccase sequence type	Closest laccase BLASTp match (Identity/Similarity,%)	E-value
Helotiaceae c7 type 1	AAL06114 *Botryotinia fuckeliana* (45/62)	6 x e^{-63}
Helotiaceae c7 type 2	AAN17298 SAP142 (62/79)	8 x e^{-108}
Dermea c14 type 1*	EAQ91617 *Chaetomium globosum* (83/89)	1 x e^{-145}
Dermea c14 type 2	AAY99671 *Cryphonectria parasitica* (63/75)	1 x e^{-101}
Dermea c14 type 3	AAY99671 *Cryphonectria parasitica* (66/79)	1 x e^{-103}
Dermea c14 type 4	CAE00180 *Melanocarpus albomyces* (43/49)	6 x e^{-63}
Oidiodendron maius c32 type 1	AAW47924 *Glomerella cingulata* (55/73)	1 x e^{-89}
Oidiodendron maius c32 type 2	AAY99671 *Cryphonectria parasitica* (52/68)	2 x e^{-69}
Hypoxylon c57 type 1	EAA68613 *Gibberella zeae* (70/83)	3 x e^{-104}
Chaetosphaeriaceae c60 type 1	AAN17298 SAP142 (97/98)	1 x e^{-159}
Chaetosphaeriaceae c60 type 2	CAD21518 *Claviceps purpurea* (49/63)	7 x e^{-78}
Sordariales c61 type 1*	EAQ91617 *Chaetomium globosum* (83/89)	1 x e^{-145}
Xylaria c63 type 1	CAE00180 *Melanocarpus albomyces* (57/71)	1 x e^{-91}
Dermataceae c79 type 1	AAL06114 *Botryotinia fuckeliana* (67/78)	4 x e^{-108}
Cryptosporiopsis c80 type 1	EAA77059 *Gibberella zeae* (52/70)	4 x e^{-50}
Ascomycota c81 type 1	EAT0499 *Phaeosphaeria nodorum* (47/63)	2 x e^{-58}
Ascomycota c81 type 2	AAL06114 *Botryotinia fuckeliana* (54/68)	3 x e^{-98}
Helotiales c82 type 1	AAL06114 *Botryotinia fuckeliana* (46/60)	6 x e^{-62}
Fulvoflamma eucalypti c83 type 1	AAW47924 *Glomerella cingulata* (55/72)	4 x e^{-87}
Pestalotiopsis c84 type 1	AAW47924 *Glomerella cingulata* (50/66)	2 x e^{-78}
Pestalotiopsis c84 type 2	EAQ91617 *Chaetomium globosum* (56/72)	6 x e^{-96}
Pestalotiopsis c84 type 3	AAW47924 *Glomerella cingulata* (45/60)	9 x e^{-64}
Glomerella acutata c85 type 1	CAD10849 *Gaeumannomyces graminis* (50/69)	7 x e^{-90}
Pilidium c86 type 1	EAA68613 *Gibberella zeae* (70/80)	1 x e^{-103}
Dothideomycetes c87 type 1	AAN17292 *Phaeosphaeria halmia* (55/68)	1 x e^{-88}
Dothideomycetes c87 type 2	EAT86325 *Phaeosphaeria nodorum* (62/73)	4 x e^{-106}
Dothidiomycetes c87 type 3	EAA68613 *Gibberella zeae* (72/85)	8 x e^{-111}
Dothideomycetes c87 type 4	AAN17289 SAP143 (47/62)	4 x e^{-75}
Ophiostoma dentifundum c88 type 1	AAN17298 SAP142 (53/67)	2 x e^{-82}
Ophiostoma dentifundum c88 type 2	XP_365454 *Magnaporthe grisea* (53/67)	1 x e^{-77}

* Sequences possess a 98% nucleotide identity.

Figure 4.4 Neighbor-joining tree of fungal laccase gene sequences from cultured fungi from ERM roots of *Rhododendron maximum*. ERM root fungi (ascomycetes) from this study provided 30 laccase gene sequences aligned with 17 additional laccase sequences from Genbank, and basidiomycetes laccase sequences as an outgroup. Tree generated from Kimura-2 parameter methods and 1000 bootstrap replicates. Values are bootstrap percentages.


Dermataceae c79 type 1
AY047482 Botryotinia fuckeliana
Ascomycota c81 type 2
Dermea c14 type 4
Helotiaceae c7 type 1
Helotiales c82 type 1
AF539829 Buergenerula spartinae
AF539816 Phaeosphaeria spartinicola
Dermea c14 type 1
Sordariales c61 type 1
AF539820 SAP162
XM_001228805 Chaetomium globosum
XM_001230067 Chaetomium globosum
Glomerella acutata c85 type 1
AB055709 Colletotrichum lagenarium
Xylaria c63 type 1
AF312321 Fusarium proliferatum
Cryptosporiopsis c80 type 1
Pestalotiopsis c84 type 2
Dothideomycetes c87 type 2
AF539821 Stagonospora sp.
AF539825 Phaeosphaeria halmia
Dothideomycetes c87 type 1
75
Hypoxylon c57 type 1
Pilidium c86 type 1
Dothideomycetes c87 type 3
XM_369674 Magnaporthe grisea
XM_390780 Gibberella zeae
Dermea c14 type 2
Oidiodendron maius c32 type 2
Dermea c14 type 3
Oidiodendron maius c32 type 1
Fulvoflamma eucalypti c83 type 1
100
Pestalotiopsis c84 type 3
AY853696 Glomerella cingulata
Pestalotiopsis c84 type 1
AF539830 Pleospora spartinae
Chaetosphaeriaceae c60 type 1
AF539831 SAP142
Helotiaceae c7 type 2
Ophiostoma dentifundum c88 type 1
Chaetosphariaceae c60 type 2
Ophiostoma dentifundum c88 type 2
AF312322 Fusarium proliferatum
100
Ascomycota c81 type 1
AF539822 Stagonospora sp.
AF539832 Phaeosphaeria halmia
Dothideomycetes c87 type 4
L10663 Agaricus bisporus
AM292415 Funalia trogii
0.1 substitutions/site

CHAPTER 5

CONCLUSIONS

We explored a plant-soil-mycorrhiza feedback with *Rhododendron maximum* in southern Appalachian forests. We found support of this hypothesis as N cycle characteristics were altered in *R. maximum* microsites due to the quantity and quality of *R. maximum* litter. Based upon recovery of ^{15}N from protein-tannin (P-T) complexes in the field, complexes derived from *R. maximum* litter tannins are more recalcitrant than those derived from hardwood litter tannins. Among mycorrhizal root types, ericoid mycorrhizal (ERM) roots of *R. maximum* were most enriched in ^{15}N from P-T complexes, suggesting that the host plant has greater access to these recalcitrant N substrates. The production of extracellular enzymes such as polyphenol oxidases by these fungi may aid in the degradation of P-T. ERM roots are colonized by a diversity of fungi, and a substantial percentage of these are not considered mutualists, suggesting that these fungi may have endophytic or loose associations with ERM roots.

Soils under *R. maximum* thickets are characterized by an accumulation of organic material and humus containing a high concentration of N (Boettcher and Kalisz, 1990). We estimated nearly 40% greater leaf and fine root litter inputs in *R. maximum* microsites compared to hardwood forest microsites, and these litter inputs are both important sources of tannins. Not only do *R. maximum* leaf and leaf litter tannins display high protein precipitation, but so do the tannins in fine roots. The ability of *R. maximum* litter tannins to precipitate protein and form recalcitrant organic complexes may be responsible for the observed increase in total N and decrease in N mineralization in these forest soils. The influence of *R. maximum* on the

availability of inorganic N, along with its reduction of photosynthetically active radiation (Clinton, 2003), may contribute to the suppression of tree seedlings in southern Appalachian forests. However, a direct test of this hypothesis is difficult given other confounding soil factors. Soil pH was significantly lower in A horizons of *R. maximum* microsites compared to hardwood microsites in our study (Wurzburger and Hendrick, 2007) and similar patterns have been reported elsewhere (Boettcher and Kalisz, 1990; Nilsen et al., 2001). Although we did not observe significant differences in soil cation concentrations between forest microsites (Wurzburger and Hendrick, 2007), differences have been observed in previous studies although they are not consistently different by soil cation, between replicate plots, nor between studies (Nilsen et al., 2001; Beier et al., 2005). If the alteration of the N cycle by *R. maximum* contributes to the mortality of seedlings, the shrub itself may acquire P-T complex N through the production of extracellular enzymes by ERM fungi. Polyphenol oxidases may contribute to the release of protein from P-T complexes, while proteases further degrade protein into bioavailable forms (Read et al., 2004). We observed elevated activities of polyphenol oxidases in soils and reciprocally placed leaf litter under *R. maximum*, but no differences in protease activities. Since proteases are broadly produced and polyphenol oxidases are less commonly produced among microbial flora (Dix and Webster, 1995), these patterns suggest differences in the composition and function of soil microbial communities between the forest microsites. The ERM fungi of *R. maximum* may also contribute to enzyme production.

Since P-T complex formation is a hypothesized mechanism behind N availability patterns, we studied the fate of P-T complex N in soils of forest microsites. Based upon a ^{15}N tracer study, the P-T complexes derived from the leaf litter of *R. maximum* microsites degraded more slowly that those derived from hardwood microsites. These results further support the

hypothesis that P-T complexes contribute to the accumulation of organic matter and the decrease in N mineralization rates in these soils. Mycorrhizal root types differed in their relative ability to acquire P-T complex N. ERM roots of *R. maximum* acquired the most P-T complex N among mycorrhizal root types per unit biomass and acquired a similar amount of ^{15}N per unit area as ectomycorrhizal (ECM) roots. Arbuscular mycorrhizal (AM) roots recovered the least ^{15}N per unit mass and per unit area. These results suggest that *R. maximum* has the greatest access to P-T complex N among forest trees and plants. Although the relative difference in ^{15}N enrichment among mycorrhizal types was subtle, the application of ^{15}N was at a low level and plants were concurrently acquiring other sources of N. Access to P-T complex N by ERM roots of *R. maximum* may be an important contribution to its N nutrition, especially when considering the potential residence time of P-T complexes in soils and the N conserving habit of this shrub. The reductions in N availability and the lesser ability for ECM and AM tree seedlings to acquire P-T complex N may contribute to their suppression.

The mechanisms by which ERM roots of *R. maximum* acquire P-T complex N are unknown, however, it is likely that ERM fungi contribute to the degradation of recalcitrant substrates and to the access of organic N. A diversity of fungi comprise the ERM root fungal community and the ecology of many of these 71 fungal taxa are unknown. We observed two known ERM symbionts and a number of possible ERM symbionts belonging to the Helotiaceae, Chaethyriales and Sebacinales. But this collection of fungi only accounted for 30% of the taxa we observed from ERM roots of *R. maximum*, and it is unknown if the remaining fungi are symbiotic or endophytic. A distinct classification between root endophytes and symbionts may not be realistic, and root-fungal relationships may operate more loosely than we previously believed (Egger, 2006). However, regardless of the ecological classification of the ERM root

fungi we observed, if these fungi actively contribute to extracellular enzyme production, they may indirectly aid the plant in acquiring nutrients contained in complex organic substrates. Because of the potential importance of polyphenol oxidases, we characterized a portion of the laccase gene sequence in ERM fungi. We observed a diversity of laccase gene sequences in ascomyceteous ERM root fungi. While the presence of a laccase gene sequence is not indicative of gene expression or enzyme production, this information is valuable for future studies. Laccase gene sequences can aid in the development of methods to determine laccase gene expression levels *in situ* and help resolve the saprotrophic role of soil and mycorrhizal fungi.

Although our research supports the idea of a plant-soil-mycorrhiza feedback with *R. maximum* the results bring to light a number of unknown aspects of the N cycle. We need to understand the mechanisms by which P-T complexes form and degrade in soils. Also, we need a greater understanding of the impact of this N feedback on the ecology of forest communities in the southern Appalachian forests. More specifically, we need to consider the importance of decreased N availability and the accumulation of P-T complex N in *R. maximum* forest microsites on the growth and survival of tree seedlings. Last, we need to consider including P-T complexes as N sources for plants and put these complex organic substrates in the context of biological soil processes in order to fully appreciate their contribution to the N cycle of forest ecosystems.

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