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BIOCHEMISTRY OF ECTOMYCORRHIZAL FUNGI: FROM FUNCTIONAL TRAITS TO ECOSYSTEM PROCESSES

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ABSTRACT

Ectomycorrhizal (EM) fungi are a group of cosmopolitan symbiotic soil fungi that colonize the finest roots of tree species and play an essential role in plant nutrition and ecosystem function. These fungi account for the majority of microbial biomass found in forest soils and as a consequence the turnover of this biomass represents a large litter input into carbon and nutrient cycles. The factors that control the decomposition of ectomycorrhizal fungi will strongly control forest litter decomposition as a whole and, thus, ecosystem nutrient and carbon cycling. Unfortunately, our understanding of these factors is poor.

It has often been suggested that chitin, a fungal cell wall polysaccharide, is a recalcitrant compound and thus a major controller of the decomposition of fungal litter. This, however, has not been explicitly examined. In Chapter 2 we examined the role of chitin in the decomposition EM fungal tissues. The study shows that chitin concentrations declined rapidly over the course of decomposition and a significant positive relationship between initial chitin concentration and decomposition was found. Together these results suggest that chitin is a labile compound relative to other compounds found in EM fungal tissue and probably does not explain the differences in decomposability of necromass across EM fungal species.

Melanin is a complex aromatic polymer found in fungal cell walls. Its concentration varies widely across fungal species. In Chapter 3 we hypothesize that variation in melanin concentration across fungal species explains a significant amount of variation in decomposition rate. To test this we examined the decomposition of EM fungal necromass of species with varying melanin concentrations in a comparative experiment. In addition, we manipulated melanin biosynthesis by inhibition in the highly melanized EM fungal species *Cenococcum*

geophilum and examined the effect on decomposition. Melanin concentration of the EM fungal necromass was negatively correlated with percent decomposition after 3 months. The inhibition of melanin in *C. geophilum* was found to increase the decomposability of its tissues. Together this suggests that melanin is likely a major biochemical control on the decomposition of EM litters and may have significant consequences on C and nutrient cycles in ecosystems.

The highly melanized and common ectomycorrhizal fungus, *C. geophilum*, is drought tolerant and abundant in water stressed habitats, yet the responsible functional traits have not been identified. In Chapter 4 we examined the role of melanin in the EM fungus *C. geophilum* under water stress by devising a series of experiments that tested the effect of the melanin biosynthesis inhibition on osmotic and desiccation stress tolerance. Melanin inhibition only had negative effects on growth when *C. geophilum* isolates were subjected to water stress but not under control conditions. This suggests that melanin production is an important functional trait that contributes to water stress tolerance of this cosmopolitan ectomycorrhizal fungus and, given the results presented in Chapter 3, likely has implications for ecosystem function.

In Chapter 5 I synthesize the research presented herein and place it in the context of prior work in a research review examining the factors influencing the decomposition dynamics of ectomycorrhizal fungal litters.

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CHAPTER 1

GENERAL INTRODUCTION

The contributions of ectomycorrhizal fungi to carbon and nutrient cycles

The function of natural ecosystems is essential to human interests. The size of carbon (C) and nutrient pools and the fluxes entering and exiting these pools determine not only ecosystem structure and productivity, they also directly impact human welfare. Globally there is more C stored in soils than in atmospheric and biotic pools so there is much interest in understanding C storage in the form of soil organic matter (SOM) to potentially mitigate rising atmospheric CO_2 by sequestering more C in soils. Because of their role as decomposers, fungi are the chief mediators of biogeochemical cycles in terrestrial ecosystems (van Der Heijden *et al.* 2008). Decomposition is the process of organic matter being broken down into simpler forms via oxidation and hydrolysis. In terrestrial ecosystems carbon and nutrients enter the soil in the form of dead tissue, or litter. The rate at which litters are decomposed is determined by the physical and chemical properties of the soil, the nature of the decomposer community, and the quality of the litter (Swift *et al.* 1979). Decomposition is fundamental to ecosystem function because it determines the availability of inorganic nutrients, which are essential to plant production (Vitousek *et al.* 1991). In addition, decomposition also determines the amounts of C sequestered in soils and returned to the atmosphere in the form of CO_2 from heterotrophic respiration.

Historically, the rate of aboveground litter production has been treated as the primary controllers of C and nutrient cycles in terrestrial ecosystems. More recently, belowground litter inputs are now considered to be just as large if not larger than aboveground inputs in some systems (Vogt *et al.* 1986; Vogt 1991; Nadelhoffer & Raich 1992; Hendrick & Pregitzer 1993).

Litter inputs resulting from the turnover of fine roots may be as large if not larger than aboveground litter inputs with more than a third of annual net primary productivity allocated to fine roots (<2 mm in diameter) (Jackson *et al.* 1997). The importance of belowground contributions to carbon and nutrient cycles has been neglected largely because our thinking has been shaped by what we are able to easily observe and access. Thus, appreciation of belowground litter inputs has been a relatively recent development coinciding with the advent of methods used to understand belowground dynamics (Vogt *et al.* 1998; Johnson *et al.* 2001; Horton & Bruns 2001; Peay *et al.* 2008). With advancements in such methods to explore belowground dynamics, we are beginning to refine ecosystem models (McCormack & Fernandez 2011).

One area of research that has benefited from such advancement is the ecology of mycorrhizal fungi. Mycorrhizal fungi are nearly ubiquitous soil organisms that form symbioses with plants in which fine roots are colonized by the fungi, which may increase nutrient uptake into the plant in exchange for carbohydrate. Approximately 90% of plant families are mycorrhizal (Smith & Read 2008). Ectomycorrhizal fungi are members of the Basidiomycota and Ascomycota and typically colonize the fine roots of trees in forest ecosystems. The benefit to host plants are well documented and include increased nutrient foraging and acquisition (Perez-Moreno & Read 2000; Baxter & Dighton 2001), water acquisition (Brownlee *et al.* 1983; Plamboeck 2007), pathogen defense (Marx 1972; Schelkle & Peterson 1997), and heavy metal tolerance (Leyval *et al.* 1997). The impact on nutrient cycling and primary productivity through their effects on plant nutrient uptake is also well documented (Read & Perez-Moreno 2003). However, direct contribution of mycorrhizal fungal litters to soils has received little attention. In forests a sizable portion (1-20%) of annual primary production (ANPP) is allocated to EM fungi

(Hobbie 2006). We are now beginning to recognize the importance of the contributions EM fungi make to forest carbon (C) and nutrient cycles as result of turnover of their tissues (Fogel & Hunt 1983; Treseder & Allen 2000; Langley & Hungate 2003; Godbold *et al.* 2006; Cairney 2012; Ekblad *et al.* 2013). Using isotopic probing, Clemmensen *et al.* (2013) have showed that a very large portion of SOM in a boreal forest system was of fungal and root origin. We therefore have a need to better understand the decomposition dynamics of belowground litter inputs and the role they play in SOM formation.

Understanding the decomposition dynamics of ectomycorrhizal fungal litters

Until recently the simplest questions regarding the decomposition dynamics of EM fungal litters remained unanswered. Using simple biochemical traits, such as lignin or N concentrations, ecologists have attempted to predict the decomposition rates of both aboveground plant litter (Melillo *et al.* 1982) and root litter (Silver & Miya 2001), although the control of root litter decomposition by lignin or N concentrations may be complicated by interactions with other root properties (Fan & Guo 2010; Goebel *et al.* 2011; Xiong *et al.* 2012) or alteration of biochemistry by mycorrhizal colonization (Langley *et al.* 2006).

Fungal tissues differ greatly in their biochemistry from those of plants and because of the intimate proximity of mycorrhizal fungi they directly alter the chemistry of roots they colonize. Fungal tissues are typically higher in N and phosphorus (P) concentrations than plant tissues, and contain cell wall compounds that are very different from those found in plants (Wessels *et al.* 1994; Bowman *et al.* 2006). It appears that in some cases N concentration explains some of the variation we find in the decomposition of fungal litter (Koide & Malcolm 2009), although this is not always the case (Wilkinson *et al.* 2011). A long held hypothesis among soil ecologists is that

chitin, a nitrogen containing fungal polysaccharide, is a recalcitrant material that results in the reduction of decomposition of fungal tissues (Treseder & Allen 2000). This has led some to surmise that the presence of chitin may be a potential mechanism reducing the decomposition of ectomycorrhizal roots (or ectomycorrhizas) and the accumulation of fungal products in the soil. Prior to the work herein, the relative rate of chitin decomposition within fungal cell walls and its effect on the overall decomposability of fungal tissues had been unknown. In Chapter 2, I explicitly determine the rate of decomposition of chitin in relation to other cell wall components.

Melanins are complex polymers found in some fungal cell walls. While the actual chemical structure of these polymers is poorly characterized, melanins are composed of phenolic or indolic monomers and result in the dark color of some fungal hyphae and spores (Butler & Day 1998).

Melanin concentration may play an important role in controlling the decomposition rate of EM fungal litter and explain some of the variation among species. Melanin concentration varies widely among fungal species, from essentially nonexistent in hyaline species to very highly pigmented, virtually black species such as *Cenococcum geophilum*. Melanin is complex and composed of aromatic monomers that require non-specific oxidative enzymes to break them down. There is some observational evidence that suggests that tissues of melanized fungi are resistant to decay (Meyer 1964; Fernandez *et al.* 2013) and the compound itself is very resistant to decomposition (Hurst & Wagner 1969; Malik & Haider 1982). However, the influence of this complex polymer on the overall decomposability of ectomycorrhizal fungal tissue has not been determined. In Chapter 3, I explicitly test the influence of melanin on the decomposition dynamics of ectomycorrhizal litters with a comparative study as well as a manipulative experiment.

Traits of ectomycorrhizal fungi: potential influence on ecological success and ecosystem process

One major area of interest in ecology concerns linking ecosystem functioning to community structure (Hillebrand & Matthiessen 2009). Ecologists are often interested in traits possessed by organisms because traits both determine suitability to habitat (and, therefore, membership in communities), and at the same time may influence ecosystem function. EM fungal communities are often extremely diverse (Walker *et al.* 2005; Tedersoo *et al.* 2006; Dickie 2007), yet our understanding of the functional diversity of these fungi, and how such diversity relates to biogeography and local abundance, is limited (Koide *et al.* 2011a).

Melanins are non-essential in primary metabolic pathways but still serve essential survival functions, particularly under stressful environmental conditions (Butler & Day 1998). Melanin production is linked to tolerance of environmental stressors including UV radiation (Singaravelan *et al.* 2008), heavy metals (Gadd & de Rome 1988) and reduced water potential (Kogej *et al.* 2006). The ubiquitous EM fungus, *Cenococcum geophilum*, is known for its ability to tolerate greater water stress than many other ectomycorrhizal fungal species (Pigott 1982; Mexal & Reid 1973). Water stress, in fact, appears to result in the dominance of *Cenococcum geophilum* in many arid and seasonally water-stressed communities (Querejeta *et al.* 2009). To date, functional traits that allow this fungus to tolerate drought and succeed in arid and seasonally dry communities have not been investigated. The production of melanin in this fungus, therefore, may be a single trait that both influences its abundance and distribution while having a large impact on biogeochemical cycling. Stressful habitats, with *C. geophilum* as a dominant member of the ectomycorrhizal fungal community, may contain an unexpectedly high concentration of C and N stored in the form of highly recalcitrant fungal litter or partial

decomposition products. With this in mind, I examine the function of melanin biosynthesis in *C. geophilum* under osmotic and desiccation stress in Chapter 4.

Toward a synthetic view of the factors that influence the decomposition dynamics of ectomycorrhizal litters

This area of research is still in its nascency. However, there is enough relevant research to begin to synthesize what we know about the decomposition dynamics of ectomycorrhizal fungal litters and, more importantly, to formulate questions for future research. In Chapter 5 I synthesize the research presented herein and place it in the context of prior work in a research review examining the factors influencing the decomposition dynamics of ectomycorrhizal fungal litters.

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CHAPTER 2

The role of chitin in the decomposition of ectomycorrhizal fungal litter

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Abstract

Ectomycorrhizal fungal tissues comprise a significant forest litter pool. Ectomycorrhizal fungi may also influence the decomposition of other forest litter components via competitive interactions with decomposer fungi and by ensheathing fine roots. Because of these direct and indirect effects of ectomycorrhizal fungi, the factors that control the decomposition of ectomycorrhizal fungi will strongly control forest litter decomposition as a whole and, thus, ecosystem nutrient and carbon cycling. Some have suggested that chitin, a component of fungal cell walls, reduces fungal tissue decomposition because it is relatively recalcitrant. We therefore examined the change in chitin concentrations of ectomycorrhizal fungal tissues during decomposition. Our results show that chitin is not recalcitrant relative to other compounds in fungal tissues and that its concentration is positively related to the decomposition of fungal tissues. Variation existing among EM fungal isolates in chitin concentration suggests that EM fungal community structure influences C and nutrient cycling.

Key words: chitin, decomposition, ectomycorrhizal fungi, soil carbon, litter recalcitrance, ecosystem function, nutrient cycling

Introduction

The decomposition of litter controls, in part, the rate at which nutrients and carbon are cycled. The cycling rates of litter materials are of fundamental importance to ecosystems. In many ecosystems the decomposition of complex organic molecules found in litter into simpler, often inorganic molecules that are available for uptake by plants controls, in large measure, the availability of nitrogen (N), which frequently determines ecosystem productivity (Chapin *et al.* 2002). Moreover, the rate of litter decomposition is one of a few factors that determines the amount of atmospheric CO_2 that can be stored in soils as organic matter (Lal 2004).

Fungi comprise 60-90% of soil microbial biomass in temperate forest ecosystems and, frequently, the majority of fungal biomass is attributable to ectomycorrhizal (EM) fungi (Fogel & Hunt 1983; Högberg & Högberg 2002). EM fungi may exert a strong, indirect influence on biogeochemical cycling by competing with saprotrophic fungi for nutrients (Gadgil & Gadgil 1971) and water (Koide & Wu 2003) and, in so doing, reducing litter decomposition. In addition, ectomycorrhizal fungi may exert important, direct effects on biogeochemical cycling. For example, significant quantities of photosynthate are delivered below-ground to ectomycorrhizal fungi. In coniferous forests, up to 22% of total fixed carbon may be allocated to ectomycorrhizal fungi (Hobbie 2006). Thus, the standing biomass of ectomycorrhizal fungi may be equivalent to that of roots (Wallander *et al.* 2004), which may comprise up to 30-50% of carbon and nutrients in terrestrial ecosystems (Jackson *et al.* 1997). Moreover, ectomycorrhizal fungi may directly influence the decomposition of the fine roots of many tree species by forming ensheathing mantles around them and by altering their chemical composition. For example, Koide *et al.* (2011) found in Pennsylvania, USA, that colonization of fine roots of *Pinus resinosa* by EM fungi either had no significant effect or significantly increased their decomposition. In a

more arid system, Langley *et al.* (2006) showed that colonization of fine roots of *Pinus edulis* by ectomycorrhizal fungi retarded their decomposition.

Because of these direct and indirect effects of ectomycorrhizal fungi, the factors that control the decomposition of ectomycorrhizal fungi will, therefore, may strongly influence forest litter decomposition as a whole and, thus, ecosystem nutrient and carbon cycling. What controls the rate at which ectomycorrhizal fungi decompose once they enter the litter pool? Some have hypothesized that fungal tissues are made more recalcitrant to decomposition by the presence of chitin (Treseder & Allen 2000, Langley & Hungate 2003, Godbold *et al.* 2006, Langley *et al.* 2006). On the other hand, some scientists have considered chitin to be a relatively labile compound (Okafor 1966; Trofymow *et al.* 1983). In actuality, we do not yet know the relative rate of chitin decomposition within fungal cell walls, which are chemically complex, nor do we know how the presence of chitin alters the decomposability of fungal tissues.

Chitin is a structural, long chain polysaccharide composed of beta linked *N*-acetylglucosamine monomers. It does not exist solitarily in the fungal cell wall. In fact, chitin is frequently a relatively minor component of fungal cell walls. In the Basidiomycota, for example, the concentration of chitin in cell walls is relatively small, between 3 and 11 % (Plassard *et al.* 1982, Markkola *et al.* 1995, Ekblad *et al.* 1998). It is usually complexed with other compounds such as glucans, proteins and melanins (Bartnicki-Garcia 1968). Such complexation could lead to different chitin decomposition dynamics in the cell wall compared to when it is pure. The first aim of our study, therefore, was to examine the decomposition of chitin within intact fungal tissues. To our knowledge no other studies have done so. We accomplished this by tracking the chitin concentrations of ectomycorrhizal fungal tissues over the course of their decomposition. If chitin is recalcitrant relative to other compounds found in fungal tissues, an increase in its

concentration would be expected as other compounds are preferentially decomposed. The second aim of the study was to determine the relationship between initial chitin concentration and the decomposability of ectomycorrhizal fungal tissues. If chitin was a recalcitrant substrate we would expect an inverse relationship between chitin concentration and decomposition rate.

Methods

Several ectomycorrhizal fungal species were isolated from sporocarps (*Rozites caperata* = *Cortinarius caperatus,* SC099; *Scleroderma citrinum,* SC031; *Suillus intermedius,* BX007; *Lactarius chrysorheus,* SC098; *Amanita muscaria,* BX008) from a *Pinus resinosa* Ait. plantation located in State College, PA, USA (40°47'43.27"N, 77°55'39.53"W). Wu *et al.* (2005) determined that the trees at this site were limited by nitrogen and phosphorus. All isolates were maintained on half strength, potato dextrose broth (Difco, BD Products, Franklin Lakes, NJ USA). Three separate cultures of each isolate were agitated gently on an orbital shaker and the fungi were allowed to grow for approximately two months. The mycelium from each culture was rinsed in distilled water and divided into three equal portions. The average dry weight of portions was *c*. 27 mg. We determined the initial chitin concentration of one portion (see below). The other two portions were dried at 70 °C, weighed and heat sealed in nylon mesh bags with 58 µm openings (SEFAR Nitex 03-58/22, Heiden, Switzerland) prior to burial in the forest floor.

The mesh bags were buried in a randomized complete block design within the F-layer of the forest floor of the same *Pinus resinosa* Ait. plantation described above on 16 September 2010. Blocks were spaced approximately 3 m from each other. During the period of the study,

the average air temperature was 19.7°C with a maximum temperature of 31.1°C and a minimum of 11.7 °C, and a total rainfall of 6.8 cm (http://www.wunderground.com). Mesh bags containing the portions of mycelia were removed to assess dry weights and chitin concentrations 7 and 28 days after burial. Upon collection all samples were dried immediately at 70°C overnight. The decomposition rate was calculated from the initial and final dry weights of each portion of mycelium.

Chitin concentrations were assayed colorimetrically using methods developed by Tsuji *et al.* 1969, Plassard *et al.* 1982 and Vignon *et al.* 1986. No single publication adequately describes the complete procedure so we present it in some detail here. In order to hydrolyze the chitin to glucosamine residues, the dried and weighed mycelium samples were added to 5 mL of 6N HCl in screw-top test tubes and maintained for 16 hours in an oven at 80°C. The pH of the hydrolysate was adjusted to approximately 3.0 by adding a 2.5 mL aliquot of 1.25M sodium acetate per 0.5 mL of the hydrolysate. In order to assay glucosamine residues, to a 1.0 mL aliquot of this solution we added 1.0 mL of 5.0% (w/v) $KHSO_4$ and 1.0 mL of 5.0% (w/v) $NaNO_2$. To establish the background level of aldehydes, to a 1.0 mL aliquot of the solution we added 1.0 mL distilled water and 1.0 mL 5.0% (w/v) $KHSO_4$. In either case, the solutions were incubated for 15 minutes with occasionally shaking, which allowed the glucosamine present in the samples subjected to $KHSO_4$ and $NaNO_2$ to deaminate. This deamination step leaves a 2,5-anhydromannose with a free aldehyde on carbon 1. After the deamination step, 1.0 mL of 12.5% (w/v) $NH_4SO_3NH_2$ was added to the solutions and shaken for 5 minutes. The free aldehyde was then reacted with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) in the presence of $FeCl_3$ to form a blue color: one milliliter of 0.5% (w/v) MBTH was added followed by a 60 min. incubation, then 1.0 mL of 0.5% (w/v) $FeCl_3$ was added, followed by a 30 min. incubation

for color development. Absorbances were determined at 653 nm. Known amounts of pure chitin (Sigma C-9752) were assayed to obtain a standard curve ($y = 1.14x + 0.012$; $R^2=0.97$), with which chitin concentrations could be calculated from the difference in absorbances between solutions containing glucosamine residues plus free aldehydes and only free aldehydes.

The effect of time, isolate and the interaction on percent litter weight remaining and chitin concentration during the course of decomposition was determined using two-way analysis of variance. The significance of mean differences were determined using Tukey's HSD test at $\alpha=0.05$. Linear regression analysis was also used to determine the relationship between initial chitin concentration and decomposition for all isolates. Statistical analyses were performed using JMP®, version 8, SAS Institute Inc., Cary, NC, USA.

Results

Overall there was a significant decline in the mass of the remaining mycelium over the course of the experiment for each of the five isolates (Table 2.1, Figure 2.1), and the rate of decline differed significantly among the five isolates as indicated by the significant interaction between time and isolate (Table 2.1).

Chitin concentration of the fungal litter declined significantly for all isolates over the course of decomposition (Table 2.2, Figure 2.2). *Post hoc* analysis revealed that the chitin concentration across isolates at t=28 days had declined significantly from the concentrations at t=0 and 14 days. Isolate was also a significant factor in the decomposition of chitin, but the interaction between isolate and time was not significant, indicating that the magnitude of decline did not depend on isolate (Table 2.2).

Across all isolates, initial chitin concentration had a significant positive relationship with

% weight loss at 28 days of the mycelia (n=15, r^2=0.29, *P*=0.037) (Fig. 2.3), suggesting that high chitin content contributes to more rapid decomposition of fungal mycelia.

Discussion

We observed significant differences in the rate of decomposition among the isolates, which supports our previous results (Koide & Malcolm 2009, Koide *et al.* 2011). A significant amount (29%) of the variability in decomposition was accounted for by variation in chitin concentration, with higher chitin concentrations associated with more rapid decomposition rates. We do not know why this was so, but it is consistent with the hypothesis that chitin can actually contribute positively to fungal litter quality and result in increased decomposability of fungal tissue. This phenomenon could be related to the presence of significant quantities of nitrogen in chitin, as nitrogen concentration has been shown to be positively related to decomposition of fungal tissue (Koide & Malcolm 2009). Furthermore, the forms of nitrogen available from the decomposition of chitin may be preferentially acquired as they may allow decomposer fungi to directly incorporate glucosamine into their cell walls while bypassing a biosynthesis step (Trofymow *et al.* 1983). Chitin can be closely associated with other compounds found in fungal cell walls including β-glucans, proteins, and melanins (Bartnicki-Garcia 1968), and these associations may further influence the decomposition of the mycelium.

There was also a significant overall decline in chitin concentration of the mycelia over the course of decomposition. This demonstrates that chitin is not particularly recalcitrant relative to other compounds found in fungal tissues. As discussed above, a significant positive relationship was found between initial chitin concentration and decomposition rate considering all isolates

together. Taken together, these results serve to refute the hypothesis that chitin increases the recalcitrance of fungal tissues to decomposition. This evidence for the relative lability of chitin is not entirely surprising because, unlike other structural polysaccharides, it contains substantial concentrations of nitrogen, which frequently limits the activities of decomposer microbes (Schimel & Weintraub 2003). Indeed, Koide & Malcolm (2009) showed that decomposition rates and N concentrations of tissues of ectomycorrhizal fungi were correlated. These results are also consistent with earlier findings that chitin is more labile than the nitrogen-free compound, cellulose (Okafor 1966; Trofymow *et al.* 1983).

In this study, significant variation among isolates in their initial chitin concentrations implies that fungal species differ in their potential to serve as sources of nitrogen in temperate forest ecosystems (Olander & Vitousek 2000). The occurrence of significant variation among isolates therefore suggests that the structure of the ectomycorrhizal fungal community may influence ecosystem nitrogen cycling.

We recognize the possibility that mycelia from liquid cultures may differ from those grown under natural conditions in ways that may influence decomposition dynamics. Nevertheless, it appears that certain aspects of fungal tissues, such as C:N ratios, are difficult to change experimentally (Koide and Malcolm 2009), possibly because the composition of cell walls, which are a major component of fungal biomass, is conserved for a given species. In addition, we examined the decomposition and changes in chitin concentration of only diffuse, vegetative mycelia, which may possess different characteristics than specialized structures such as sporocarps or rhizomorphs. Plant litter decomposition may exhibit separate phases during which various components are degraded (Berg 2000). It is not clear whether fungal mycelium also exhibits distinct decomposition phases, but we point out that in this study decomposition of the

mycelia was between approximately 20 and 40%. Decomposition was not merely in the early phases. Thus, while we cannot say with certainty what will happen in a longer-term study, our results suggest that over a large fraction of the decomposition process, chitin does not appear to be particularly recalcitrant.

Conclusions

Chitin does not appear to be particularly recalcitrant relative to other compounds found in fungal tissues. Indeed, higher concentrations of chitin in fungal cell walls were associated with higher rates of decomposition. In some ecosystems, therefore, fine root decomposition is not likely to be retarded as a consequence of the presence of chitin in the fungal cell walls comprising the mycorrhizal mantle, and higher chitin concentrations in ectomycorrhizal fungal litter may result in lower equilibrium soil organic matter concentrations, all else being equal. Moreover, variation among ectomycorrhizal fungal species in chitin concentration may result in variation in their effectiveness as sources of nitrogen from their decomposing litter. The structure of ectomycorrhizal fungal communities thus has the potential to influence carbon and nitrogen cycling, key ecosystem functions.

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helpful comments.

Figures and Tables

Table 2.1. Results of the two-way ANOVA tests for mean percent mass remaining with isolate, time, and their interaction as explanatory variables.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob.>F
Model	14	20544.421	1467.46	40.7913	<0.0001*
Error	30	1079.243	35.97		
C. Total	44	21623.664			
Effects test					
Isolate	4	3033.087		21.0779	<0.0001*
Time	2	15917.948		221.2377	<0.0001*
Isolate*Time	8	1593.386		5.5365	0.0002*

Figure 2.1. Mean percent mass remaining of mycelia of each of the five species of ectomycorrhizal fungi at 0, 14 and 28 days. Vertical bars indicate ± 1 SEM.

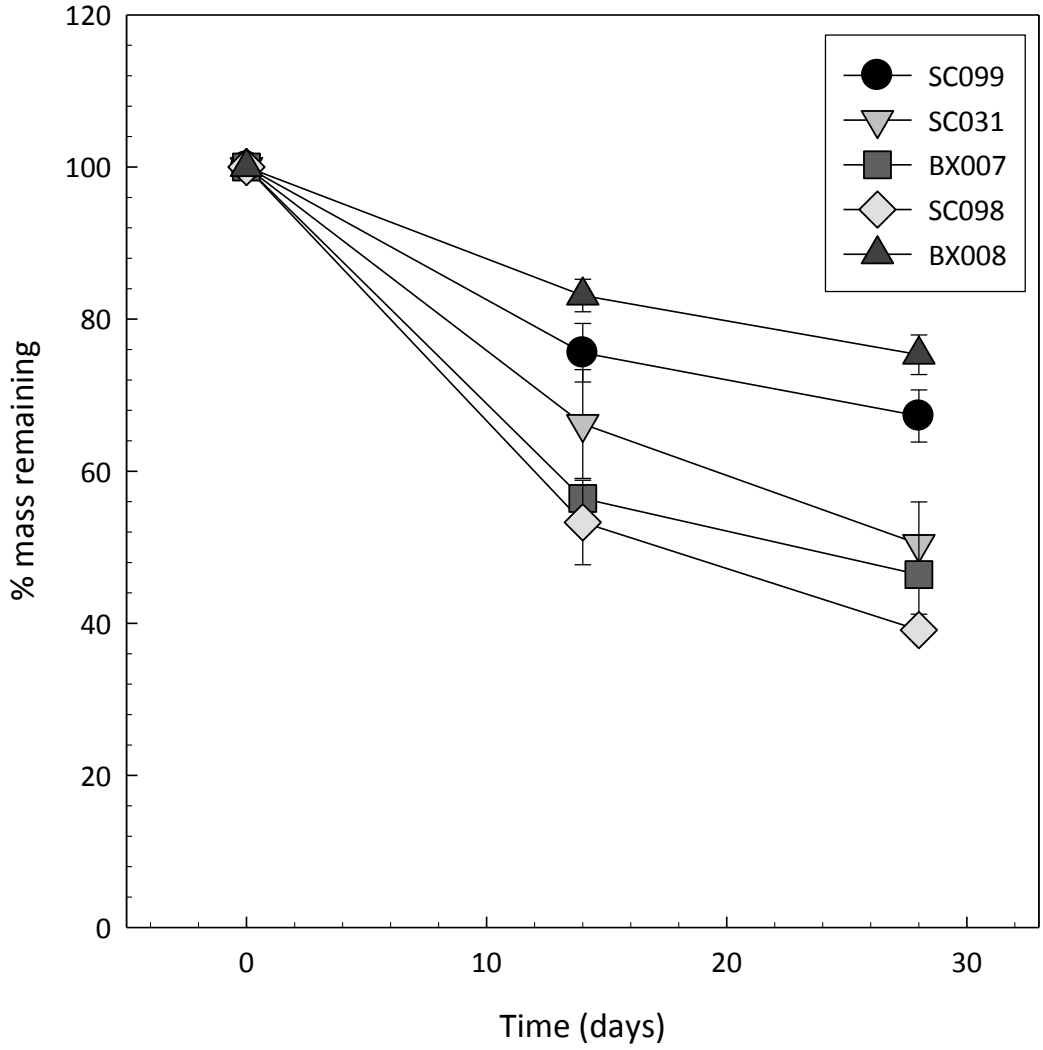


Table 2.2. Results of the two-way ANOVA tests for mean chitin concentration with isolate, time, and their interaction as explanatory variables.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob.>F
Model	14	10606.059	757.576	5.0953	<0.0001*
Error	30	4460.446	148.682		
C. Total	44	15066.505			
Effects test					
Isolate	4	4229.3657		7.1115	0.0004*
Time	2	4805.4384		16.1602	<0.0001*
Isolate*Time	8	1571.2549		1.321	0.2712

Figure 2.2. Mean chitin concentrations (mg g^{-1}) for decomposing mycelia of each of the five species of ectomycorrhizal fungi at 0, 14 and 28 days. Vertical bars indicate ± 1 SEM.

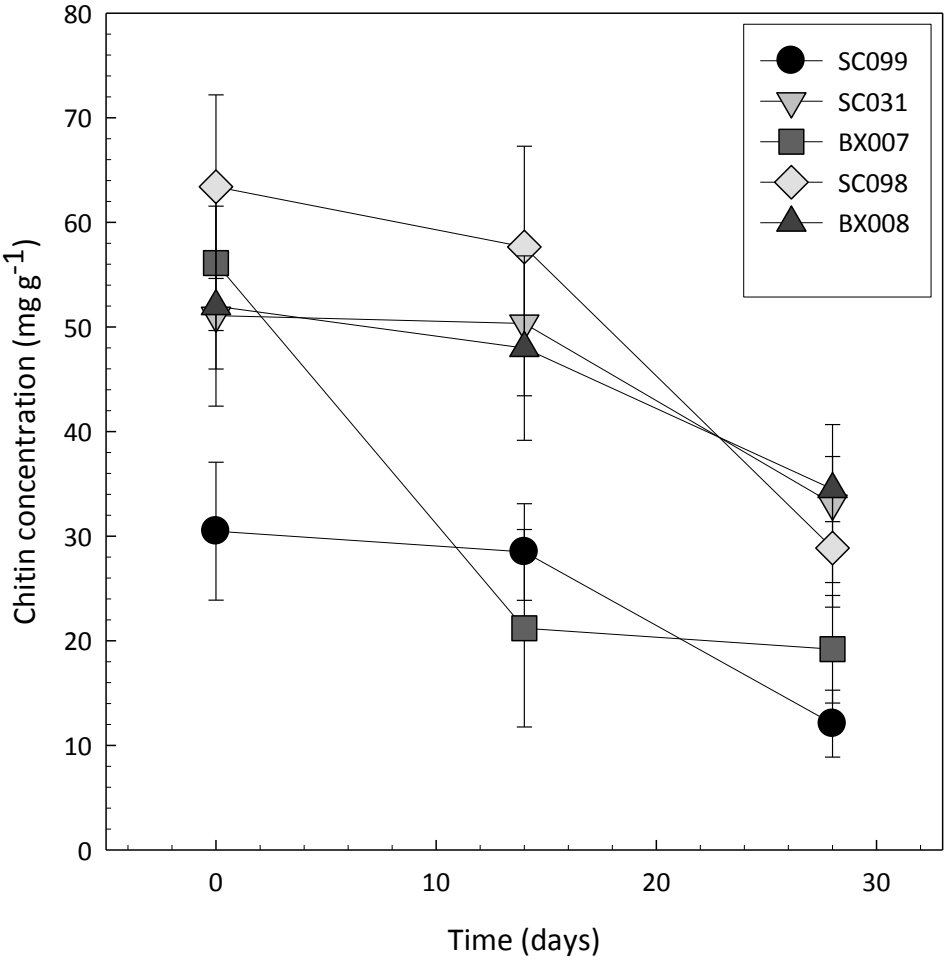
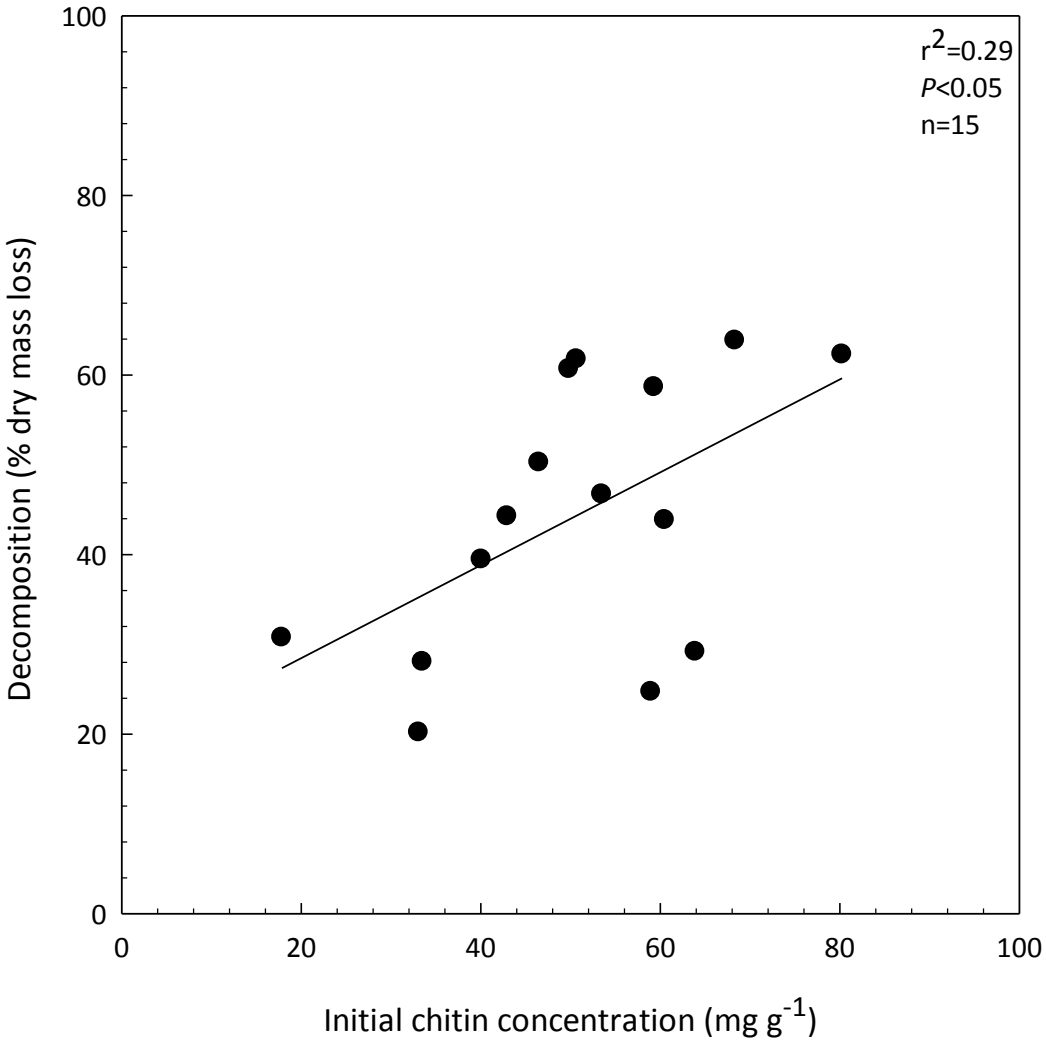


Figure 2.3. The relationship between initial chitin concentration of EM fungal mycelia and their decomposition (% mass loss) at 28 days. y = 5.17x + 0.181, n=15, r^2= 0.29, *P*< 0.05.



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CHAPTER 3

Is melanin a major controller of the decomposition of ectomycorrhizal fungal litter?

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Keywords: carbon cycling; *Cenococcum geophilum*; decomposition; ectomycorrhizal fungi; nutrient cycling; melanin

In review- *Ecology*

Abstract

The turnover of ectomycorrhizal (EM) fungal biomass is now recognized as a large and important input into ecosystem carbon (C) and nutrient cycles. Thus, examining the decomposition dynamics of EM fungal litters will likely help improve understanding of these cycles. Melanin may exert some control of the decomposition of fungal litters because it is a recalcitrant, complex aromatic polymer found in fungal cell walls and it varies widely in concentration across fungal species. In a comparative study we examined the influence of melanin on decomposition of EM fungal necromass with varying melanin concentrations. In addition, we inhibited melanin biosynthesis in the highly melanized EM fungal species *Cenococcum geophilum* and examined the effect on decomposition of its necromass. Initial melanin concentration was negatively correlated with decomposition (% mass loss) of the EM necromass and when melanin synthesis was inhibited in *C. geophilum*, its necromass decomposed significantly more rapidly. We conclude that melanin concentration is likely a major biochemical control on the decomposition of EM fungal litters and may have significant consequences on C and nutrient cycles in ecosystems.

Introduction

As the primary decomposers of plant litter and soil organic matter (SOM), fungi are drivers of carbon (C) and nutrient cycles in ecosystems. Additionally, the fungi themselves are now appreciated as important inputs into these cycles, particularly the necromass resulting from the death of mycorrhizal fungi (Langley & Hungate 2003; Cairney 2012; Ekblad *et al.* 2013). Ectomycorrhizal (EM) fungi are estimated to have up to 20 % of net annual primary productivity allocated to them by their hosts (Hobbie 2006) and, as a result, comprise the majority of microbial biomass in forest soils (Fogel & Hunt 1983; Högberg & Högberg 2002). Therefore, the turnover of EM fungal tissues represents a large flux into soil carbon and nutrient cycles (Ekblad *et al.* 2013). Recent evidence from carbon isotope probing suggests that much of the SOM in a boreal forest originates from fungi and roots rather than aboveground plant tissues (Clemmenssen *et al.* 2013). In order to improve our understanding of C and nutrient cycling in soils, an important line of research focused on understanding the processes governing the production and turnover of EM fungal litter has emerged (Cairney 2012; Ekblad *et al.* 2013).

The decomposition of EM fungal litters likely has significant influence on SOM formation. Therefore, understanding the factors that control this decomposition is of great interest (Langely & Hungate 2003). As with plant litters, the decomposability of EM fungal necromass varies across species (Koide & Malcolm 2009; Wilkinson *et al.* 2011) but the mechanisms causing this variation remain poorly understood. The fungal cell wall component, chitin, has long been thought to contribute to the recalcitrance of fungal tissues (Treseder & Allen 2000). However, there is growing evidence which suggests that chitin is relatively labile (Fernandez & Koide 2012; Drigo *et al.* 2012; Zeglin *et al.* 2012). Examining biochemical components of fungal cell walls that vary widely in concentration across EM fungal species is

more likely to explain the differences we observe in their decomposition rates. For plant litters, lignin concentrations, which vary widely among species, frequently appear to control decomposability (Meentemeyer 1978; Melilo *et al.* 1982). Melanin may do the same for fungal litters.

Melanins are a group of complex polymers found in fungal cell walls. While the actual chemical structure of these polymers is poorly characterized, melanins are composed of phenolic or indolic monomers and result in the dark color of some fungal hyphae and spores (Butler & Day 1998). Fungi produce four types of melanin that are differentiated based on their biosynthetic pathway. They include 3,4-dihydroxyphenylalanine (DOPA) and γ-glutaminyl-4-hydroxybenzene (GDHB), both of which are present in Basidiomycetes, and 1,8-dihydroxynaphthalene (DHN) and catechol melanin, which are produced primarily by Ascomycetes (Butler & Day 1998a). All melanin types share a complex and irregular chemical structure composed of aromatic monomers that are acid insoluble and gives fungal cell walls a dark brown to black color (Butler & Day 1998). Melanin production in fungi has been shown to have protective properties from UV radiation (Singaravelan *et al.* 2008), osmotic stress (Kogej *et al.* 2006; Fernandez & Koide 2013), heavy metal stress (Gadd & de Rome 1988) and antagonistic interactions (Nosanchuk & Casadevall 2003). There are a number of reasons why melanin concentration may play an important role in controlling the decomposition rates of EM fungal litter and explain some of the variation found across species. First, melanin concentration varies widely across fungal species, from hyaline species that produce very little to very highly pigmented species such as *Cenococcum geophilum*. Second, there is evidence that melanin is highly resistant to decomposition. The chemical structure of melanin is highly complex and composed of aromatic monomers requiring non-specific oxidative enzymes to break them down.

Soil microcosm studies have shown that melanized cell walls decompose more slowly than hyaline cell walls and melanin fractions decompose more slowly than other fungal cell fractions (Hurst & Wagner 1969; Malik & Haider 1982). Meyer (1964) suggested that the ectomycorrhizas of the highly melanized fungus *Cenococcum geophilum* may be resistant to decomposition and may accumulate in the soil, thus significantly contributing to SOM. Fernandez *et al.* (2013) provided supporting evidence for this using minirhizotron imaging. They found that ectomycorrhizas of *C. geophilum* persisted in the soil 4-10 times longer than those of other species. These evidences suggest, therefore, that melanin concentration is likely to be a major controller of the decomposition of EM fungal litter and that highly melanized species, such as *C. geophilum,* contribute disproportionately to SOM formation.

The purpose of this study was to answer the following questions: 1) Does melanin concentration negatively influence the decomposition of EM fungal necromass? 2) How much variation among species of ectomycorrhizal fungi in decomposition rate can be explained by initial melanin concentration? 3) When melanin biosynthesis is reduced in the heavily melanized EM fungus *C. geophilum*, does the decomposability of its necromass increase? To address these questions we conducted a litter bag study examining the relationship between melanin concentration of various EM fungal isolates and the decomposition rate of their necromass. In addition, a manipulative experiment was conducted in which the effect of melanin biosynthesis inhibition on decomposition was determined in three isolates of the highly melanized EM fungus *Cenococcum geophilum*.

Methods

Study site

The study was conducted in an approximately 80 year old red pine (*Pinus resinosus* Ait.) plantation located in State College, Pennsylvania, USA (40°47' 43.27" N, 77° 55' 39.53" W). The site has a well-developed O-horizon comprising a litter (L) layer, fermentation (F) layer, and humified (H) layer. The mineral soil is classified as a Morrison sandy loam consisting of a thin eluviated A-horizon and a well-defined sandy B-horizon. Both experiments were conducted over the course of a three month period from April 18, 2013 to July 17, 2013. The mean daily maximum and minimum air temperatures were 23.07 °C and 12.85 °C, respectively, and 41.75 cm of total precipitation fell during the course of the study (data from National Climate Data Center, NOAA; http://www.ncdc.noaa.gov).

Experiment 1

The isolates of EM fungi were selected to capture a range of melanin concentration based on visual assessment of their pigmentation. Five isolates of Basidiomycetes including *Lactarius chrysorrheus* (SC016), *Scleroderma citrinum* (SC200), *Tylopilus felleus* (SC201),*Thelephora terrestris* (TtTor103), *Lactarius* sp. (SC202) and three isolates of *Cenococcum geophilum* (SC032, CgTor109, CeG02) were used in this experiment. The EM fungal isolates were cultured from sporocarps (or sclerotia for the *C. geophilum* isolates) that were collected from the study site described above (SC016; SC032; SC200; SC201; SC202), Southern Sweden (TtTor103; CgTor109), and Mt. Fuji, Japan (CeG02). All isolates were maintained on half-strength potato dextrose agar (PDA) prior to initiating the experiment.

Fungal biomass of each isolate was grown by taking 5 mm diameter plugs from agar cultures and placing them in 50mL of half-strength potato dextrose broth (PDB; Difco, BD Products, Franklin Lakes, New Jersey, USA) agitated gently on an orbital shaker (n=3 per

isolate). Colonies were harvested and washed with distilled water and were split into three subsamples. Separate subsamples were used for the analysis of melanin concentration, the analysis of N concentration, and for inclusion in the decomposition study.

A quantitative colorimetric assay was used to measure the initial melanin concentrations of the necromass used in the decomposition experiment (Butler & Lachance 1986; Fredrick *et al.* 1999). This method relies on the strong binding of the dye Azure A (Adrich #861049) to melanin. In this method melanin is quantified by measuring the difference in absorbance of the dye solution before and after exposure to the melanin. Fungal hyphae with high melanin concentrations will produce a relatively large change in the absorbance of the dye solution. The Azure A dye solution was made by dissolving the dye in 0.1 M HCl. To ensure there was no undissolved dye remaining, the solution was filtered through a 0.45 μm nitrocellulose membrane (Pall #66278). The dye solution was diluted until an absorbance of 0.665 at 610nm was achieved. A standard curve was constructed using known amounts of stock melanin that had been isolated from *Cenococcum geophilum* mycelium by acid hydrolysis. Melanin is acid insoluble, which allows for the selective hydrolysis of the other cell components with a strong acid while leaving the melanin intact. Mycelium from *Cenococcum geophilum* isolates were placed in 6 M hydrochloric acid at 80 °C for 4 days. After acid hydrolysis the solution and undissolved material were filtered (Fisher #09-803-6C) and the solids (melanin) were washed several times with deionized water. The stock melanin was then lyophilized and stored in a -20 °C freezer until needed. To generate the standard curve known values of the stock melanin were placed in 3 mL of Azure A solution and incubated for 90 minutes. The stock melanin and dye solution were then filtered through a 0.45 μm syringe tip filter (Adrich # Z672882) and the absorbance of the filtrate was measured at 610 nm using a spectrophotometer. A standard curve

was generated using the change in absorbance after incubation with the stock melanin (melanin (g) = Δ abs *-0.0103).

All fungal samples were dried at 70 °C overnight and weighed before analysis. Subsamples reserved for initial melanin concentration analysis were placed in 3 mL of the Azure A dye solution and were allowed incubate for 90 minutes. The sample and solution were then filtered through a disc 0.45 μm syringe tip filter. The absorbance of the filtrate was then measured using a spectrophotometer at 610 nm. The change in absorbance was then used to calculate the melanin content of the sample based on the standard curve. A second subsample was reserved for measuring N concentrations using an elemental analyzer (EA 1110, CE Instruments, Thermo Electron Corp). There was not enough tissue for measuring N for the SC202 isolate, so it was not included in the analysis. Finally, the remaining subsample was placed in a mesh litter bag with 58 μm openings (SEFAR Nitex 03-58/22; Sefar, Heiden, Switzerland) that were then heat sealed. A randomized complete block design was used with three blocks approximately 3 m apart from each other. Litter bags of each isolate were placed randomly in each block in the F-layer of the soil of the *Pinus resinosa* plantation described above. The samples were harvested after 3 months and decomposition rates were calculated from the initial and final dry weights. In order to determine the influence of melanin and N concentrations on the decomposition of the EM fungal necromass we first used simple linear regression analyses. Later, we constructed a multiple linear regression model with both initial melanin and N concentrations as predictors of decomposition (percent mass loss) of the fungal necromass after three months. All statistical analyses were conducted with JMP Pro 10 (SAS Institute Inc., Cary, NC, USA).

Experiment 2

Melanin biosynthesis was inhibited in the three *C. geophilum* isolates (SC032, CeG02, CgTor109) using the DHN melanin inhibitor, tricyclazole 5-methyl-1,2,4-triazolo(3,4,b)-benzothiazole (tricyclazole). Half-strength PDB was amended with the inhibitor at a concentration of 100 μg ml^{-1}. Tricyclazole inhibits key reductase enzymes critical to the production of DHN melanin without having adverse effects on growth under normal conditions (Butler & Day 1998a). Tricyclazole is insoluble in water so, in order to dissolve the inhibitor in the media, it was first dissolved in a small aliquot of ethanol (750 μL) before being added to the PDB (<0.0001%). The same amount of ethanol was added to the control cultures. At this concentration the inhibitor does not completely halt melanin biosynthesis but does significantly reduced giving the necromass of *C. geophilum* a dark-brown rather than a jet black appearance (Fernandez & Koide 2013). Unfortunately we were unable to quantitate this change in melanin concentrations because the assay was found to be unreliable when assessing the tricyclazole treated mycelia. However, we are certain that tricyclazole effectively inhibits melanin synthesis in *C. geophilum* based on visual inspection and from previous studies showing decreased melanin deposition in the cell wall (Fernandez & Koide 2013). The necromass from each replicate (n=3 per treatment and isolate) was split into 3 subsamples and were dried to measure dry mass. Dry tissues were placed in a mesh litter bag with 58 μm openings (SEFAR Nitex 03-58/22; Sefar, Heiden, Switzerland) that were then heat sealed. A randomized complete block design was used with three blocks approximately 3 m apart from each other. Litter bags of each isolate and treatment were placed randomly in each block in the F-layer of the soil of the *Pinus resinosa* plantation described above. The samples were harvested once every 4 weeks for 3 months and decomposition rates were calculated from the initial and final dry weights.

A three-way ANOVA model with isolate, melanin inhibition treatment, time and their interactions was used to determine significant effects on decomposition of the necromass. All statistical analyses were conducted using JMP Pro 10 (SAS Institute, Cary, NC, USA).

Results

Experiment 1

Melanin concentrations of the isolates (Figure 3.2) corresponded well with their appearance. The three *Cenococcum geophilum* (Ascomycete) isolates had the highest melanin concentrations that ranged from 154 to 300 mg g^{-1}. The Basidiomycete isolates had lower melanin concentrations that ranged from 32.3 to 190 mg g^{-1}. We found no significant effect of block. There was considerable variation in the % decomposition of the necromass across the isolates (one-way ANOVA; $F_{7,16}$ =5.93; P=0.0016). A significant negative relationship was found between the initial melanin concentration and the percent mass loss after 3 months (n=24; P=0.0219; R^2=0.22; Figure 3.3A). One replicate of SC016 had extremely low decomposition (12.90 % mass loss after 3 months), which was unusual when compared to the other replicates for this isolate. We suspected that this data point was an outlier and when removed the amount of variation in decomposition explained by initial melanin concentration improved greatly (n=23; P= 0.0003; R^2=0.48; Figure 3.3A). Upon examining the N concentration it was revealed that this replicate possessed an unusually low N concentration (1.41 %) relative to the other replicates, which had a mean of 2.07 %.

The initial N concentration of the necromass was positively related to the decomposition rate (n=21; P=0.0064; R^2=0.33; Figure 3.3B). Since litter quality is often determined by carbon

quality and N content, we constructed a model that included initial N and melanin concentrations and their interaction as factors explaining decomposition (% mass loss after 3 months). We found no multicollinearity between initial N and melanin concentrations (n=21; *P*=0.56; Figure 3.S1). The multiple linear regression model was a superior at explaining the most variation in decomposition (n=21; *P*=0.0011; R^2=0.53). When the decomposition rate constants were plotted against the initial melanin (mg g-1) : initial N (%) ratios we found a significant nonlinear negative relationship (n=21; P< 0.0001; R2=0.74; Figure 3.4).

Experiment 2

There was a significant time effect (Table 3.2) where percent mass loss of the necromass increased at each sampling (Figure 3.4). The melanin inhibition treatment significantly increased the decomposition rate of the necromass relative to the controls in all three of the *C. geophilum* isolates at all three sampling points (Table 3.2; Figure 3.4). Melanin-inhibited necromass experienced rapid decomposition during the first month relative to the control with mean % decomposition twice that of the control across isolates. This difference in percent mass loss appeared to lessen by the third month of decomposition, though there was no significant interaction between 'Time' and 'Melanin Inhibition' factors (Table 3.2).

Discussion

In this series of experiments we showed that melanin concentration appears to be a significant control on the decomposition of EM fungal tissues. This was demonstrated by examining decomposition rates of EM necromass of EM fungi varying naturally in melanin

concentration in Experiment 1, which was further supported by the results from Experiment 2 in which we manipulated melanin concentration experimentally with a melanin synthesis inhibitor in three *C. geophilum* isolates. In addition, we found that initial N concentration was also a significant predictor of decomposition; initial N concentration was positively related to the percent mass loss of the necromass, which supports the findings of Koide & Malcolm (2009). The multiple regression model with initial melanin and N concentrations as predictors explained more variation in decomposition rates than each of the univariate models. These findings are consistent with patterns seen in the decomposition of plant litters that show that carbon quality and N content are both important biochemical controls on decomposition rates (Mellilo *et al.* 1982). We therefore argue that melanin is a fungal analogue to lignin in terms of its influence on the decomposition of fungal tissues by reducing the carbon quality. The chemical structure of melanin, like lignin, is irregular, complex and composed of aromatic monomers and its decomposition requires the use of oxidative enzymes. In fact, the very same oxidative enzymes that decomposers use to break down lignin have been demonstrated to be effective in breaking down melanin (Butler & Day 1998b). Like lignin in plants, melanin also likely inhibits the decomposition of other cell wall components for three reasons. First, higher concentrations of relatively recalcitrant compounds reduce the overall substrate quality of the litter, which reduces the return on investment in extracellular enzymes. Second, the fungal cell wall is composed of compounds that are enmeshed with each other (Feofilova 2010). Melanin likely physically protects other cell wall compounds like chitin, β-glucans and proteins from extracellular enzymes (Kuo & Alexander 1967). Finally, melanin has been shown to actually permanently inhibit extracellular enzymes involved in decomposition of protein, chitin and β-glucans (Bull 1970). While the melanin assay used in this study is not sensitive to melanin type and therefore

we were unable to examine differences across fungal melanin types, the chemical structure of all melanin types are complex and aromatic in nature (Butler & Day 1998a) and would all require the implementation of the same oxidative enzymes to break them down. Therefore, concentration rather than melanin type is likely to be a more important factor in terms of litter decomposability.

Ectomycorrhizal fungi influence the decomposition of the fine roots they colonize, a very large litter input in ecosystems, by altering their biochemistry (Langley & Hungate 2003). However, the results from these studies have been conflicting. Langley *et al.* (2006) showed that EM fungal colonization reduced the decomposition of fine roots. Conversely, Koide *et al.* (2011) found that most EM fungal species did not significantly reduce the decomposition of fine roots and in one case, EM fungal colonization actually increased the decomposition relative to non-mycorrhizal fine roots. Melanin concentration is a factor that may explain the difference found in these studies. Langley *et al.* (2006) examined ectomycorrhizas that were primarily formed by fungi in the genus *Geopora,* which appear to have a relatively high level of melanization (*personal communication,* Catherine Gehring), while Koide *et al.* (2011) examined ectomycorrhizas that were formed by hyaline Basidiomycetes. Furthermore, results presented in Fernandez *et al.* (2013) corroborate these claims with evidence of exaggerated persistence times of *C. geophilum* ectomycorrhizas relative to ectomycorrhizas of other, less melanized EM fungi. This recalcitrance may also have significant implications on SOM formation, particularly in communities that are dominated by *C. geophilum*. It is not uncommon for this fungus to dominate EM fungal communities (Izzo *et al.* 2005; Koide *et al.* 2005) and, in some cases, *Cenococcum geophilum* can completely dominate the community with relative frequencies as high as 97% (Trocha *et al.* 2012). A promising approach to understanding the influence of

melanin of EM fungal litter on SOM formation might involve comparing communities in which *C. geophilum* is found in high frequency with those in which it is rare.

Beyond being a structural component of the cell wall, melanin has been shown to be an important protectant from environmental stressors (Gadd & de Rome 1988; Nosanchuk & Casadevall 2003; Kogej *et al.* 2006; Singaravelan *et al.* 2008; Fernandez & Koide 2013). Thus, environmental factors may influence the occurrence and abundance of melanized EM fungi and, as a consequence, biogeochemistry. An intriguing example of this may be *Cenococcum geophilum*, an EM fungal species (or species complex) found in EM fungal communities that are arid or experience seasonal water stress (Pigott 1982; Querejeta *et al.* 2009). Fernandez & Koide (2013) showed that melanin production was a key trait in tolerating water stress for this species. Thus, the trait is involved in tolerance to water stress which is likely to increase its fitness in water stressed communities and consequently influence the C and nutrient cycling in these ecosystems. Melanin concentration, therefore, may act as both a response trait and an effect trait (Koide *et al.*, 2013).

Conclusions

Melanin concentration, as lignin in plant litter, appears to be a major biochemical controller of decomposition of EM fungal litter. Thus, highly melanized species, such as *C. geophilum*, are likely to contribute disproportionately to SOM formation ecosystems because of their resistance to decomposition. Going forward, we argue that measuring the influence of traits of EM fungi, such as melanization, which affect important ecosystem processes such as SOM formation and nutrient dynamics, is of utmost importance if we are to fully understand biogeochemical cycling in forest ecosystems.

Acknowledgments

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Figures and Tables

Figure 3.1. Photographs of selected isolates grown on potato dextrose agar that were used in the study A. *Tylopilus felleus* (SC201), B. *Lactarius chrysorrheus* (SC016), C. *Scleroderma citrinum* (SC200), D. *Lactarius* sp. (SC202), E. *Thelephora terrestris* (TtTor103), F. *Cenococcum geophilum* (CgTor109).

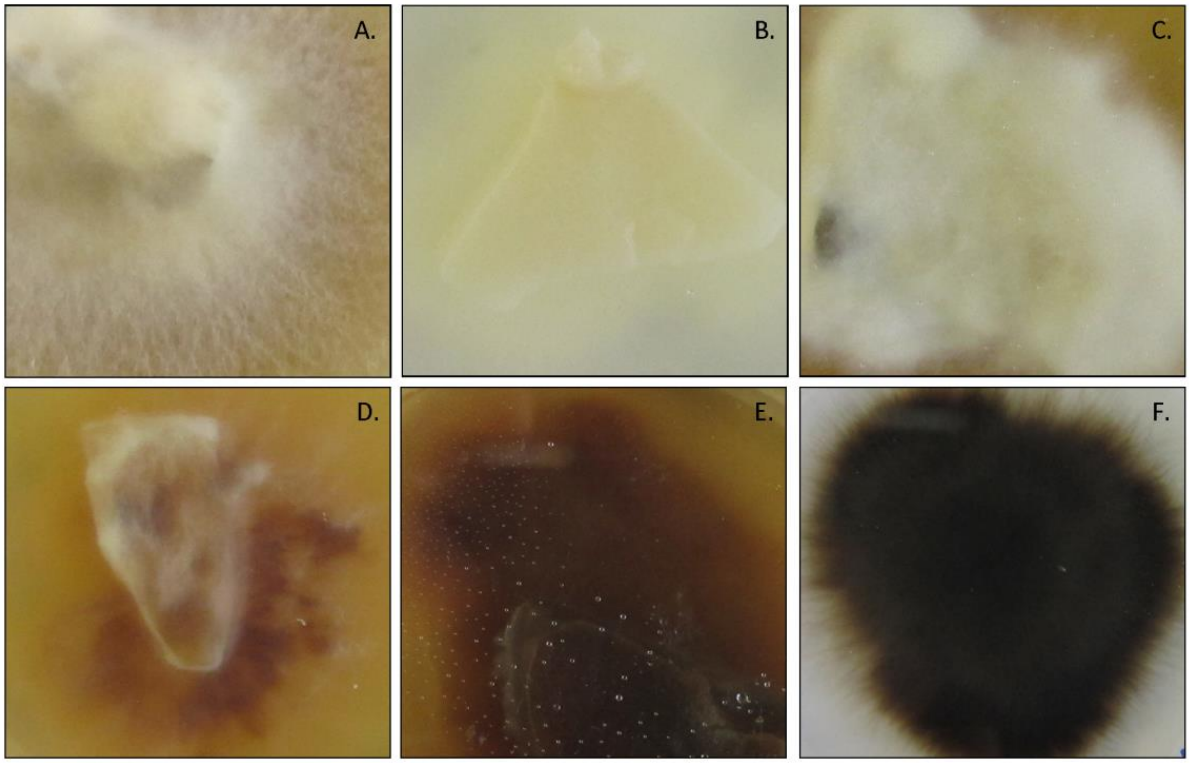


Figure 3.2. Mean melanin concentrations of the isolates used in Experiment 1 as determined by Azure A dye colorimetric assay (n= 3 per isolate). Vertical bars indicate ±SEM. Mean contrasts were determined to be significant at the α=0.05 level using Tukey's HSD test and are indicated with letters.

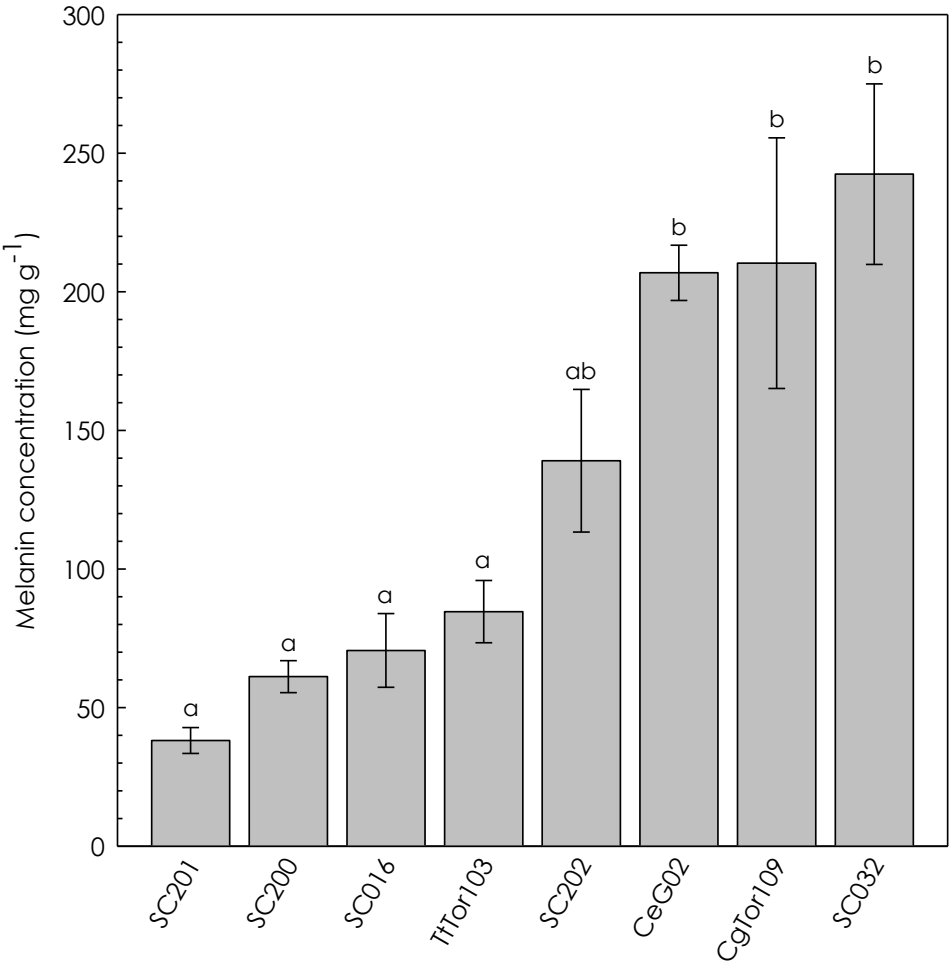


Table 3.1. Mean (±SE) carbon (C), nitrogen (N) concentrations (% mass) and C:N ratios for the isolates used in Experiment 1. Isolate SC202 was excluded because there was not enough tissue for the analysis.

Isolate	C	N	C:N
SC016	37.34 (0.46)	1.85 (0.23)	20.83 (2.71)
SC200	41.35 (0.54)	4.14 (0.06)	10.00 (0.22)
SC201	36.65 (3.21)	3.50 (0.31)	10.47 (0.10)
TtTor103	37.78 (1.91)	3.62 (0.25)	10.60 (1.20)
CeG02	44.64 (1.17)	3.02 (0.15)	14.88 (1.10)
CgTor 109	47.36 (0.74)	3.02 (0.33)	16.14 (2.04)
SC032	46.71 (0.68)	3.30 (0.38)	14.54 (1.71)

Figure 3.3. A. The relationship between initial melanin concentration and decomposition (% mass loss) of EM fungal necromass in Experiment 1. after three months. Best fit line excluding the SC016 replicate c (dashed) Decompostion (% mass loss) = -0.00121 * Melanin concentration + 0.705; n=23; *P*=0.0003; R^2=0.48. Best fit line including the SC016 replicate c (solid) Decompostion (% mass loss) = -0.00092 * Melanin concentration + 0.646; n=24; *P*=0.0219; R^2=0.22. **B.** The relationship between initial nitrogen concentration and decomposition (% mass loss) of EM fungal necromass in Experiment 1. after three months. Best fit line Decompostion (% mass loss) = 0.125 * Nitrogen concentration (%) + 0.107; n=21; *P*=0.0064; R^2=0.33.

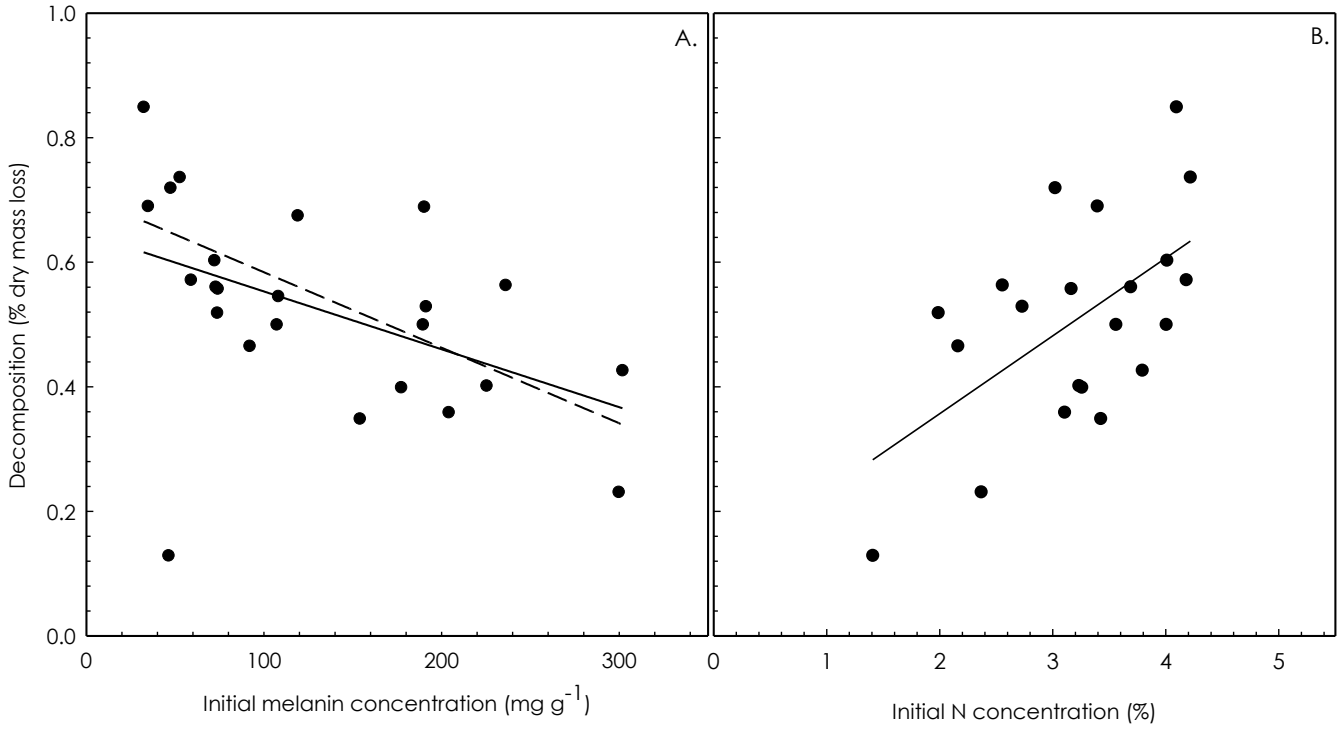


Figure 3.4. Nonlinear relationship between Initial Melanin (mg g^{-1}) : Initial N (%) and the decomposition rate constant (k). Decompostion rate constant = 0.117 + (3.52/melanin : N); n=21; P<0.0001; R^2=0.74.

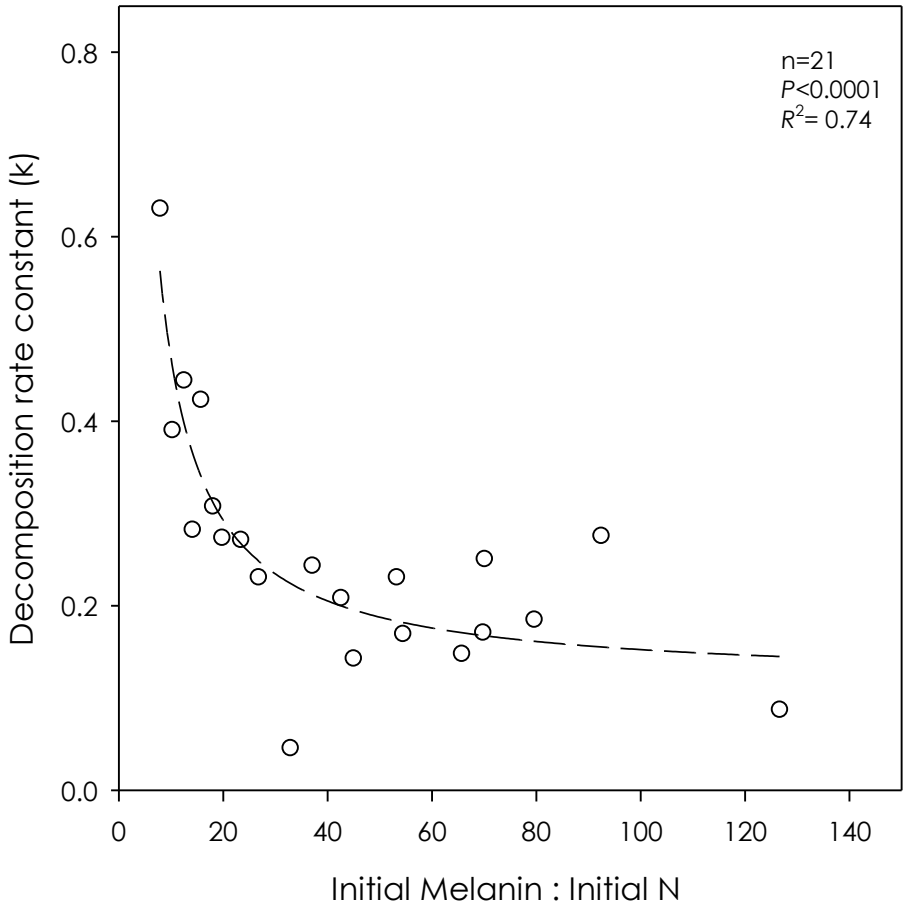
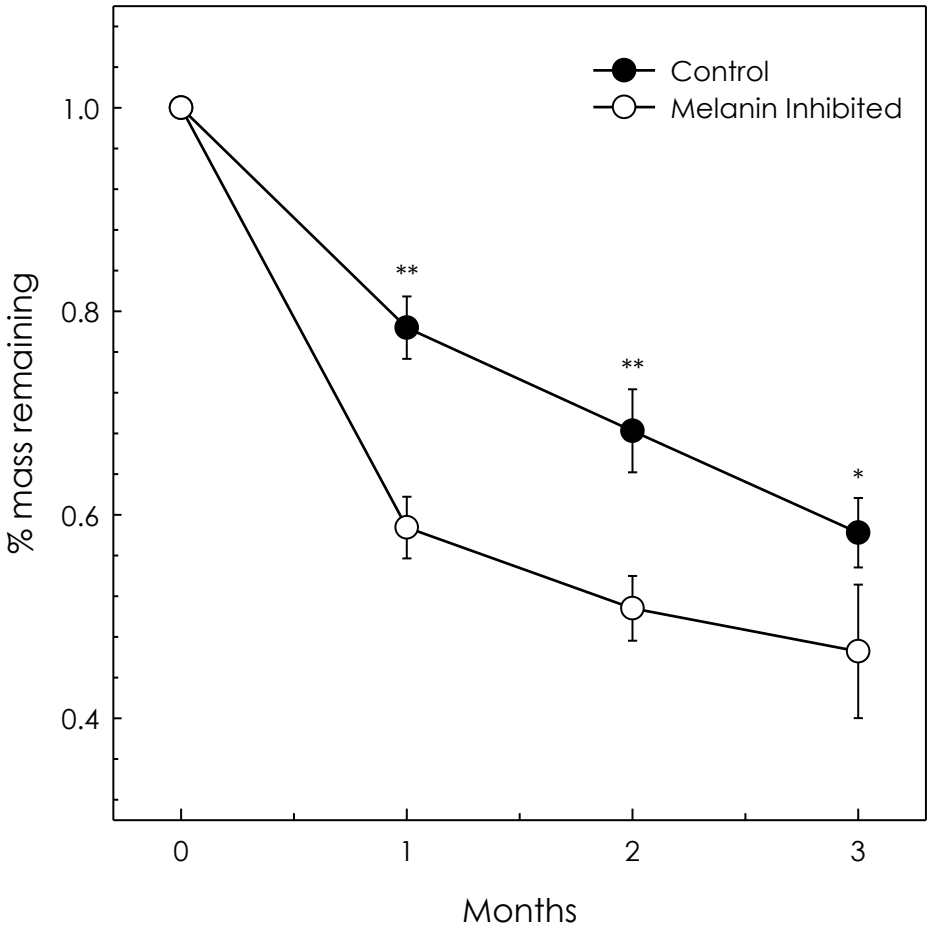


Table 3.2. Results from the three-way ANOVA tests for mean percent mass loss with Isolate, Melanin Inhibition Treament, Time and their interactions as explanatory variables. Asterisks indicate statistical significance at the $\alpha<0.05$ (*), $\alpha<0.01$(**), and $\alpha<0.001$(***) level.

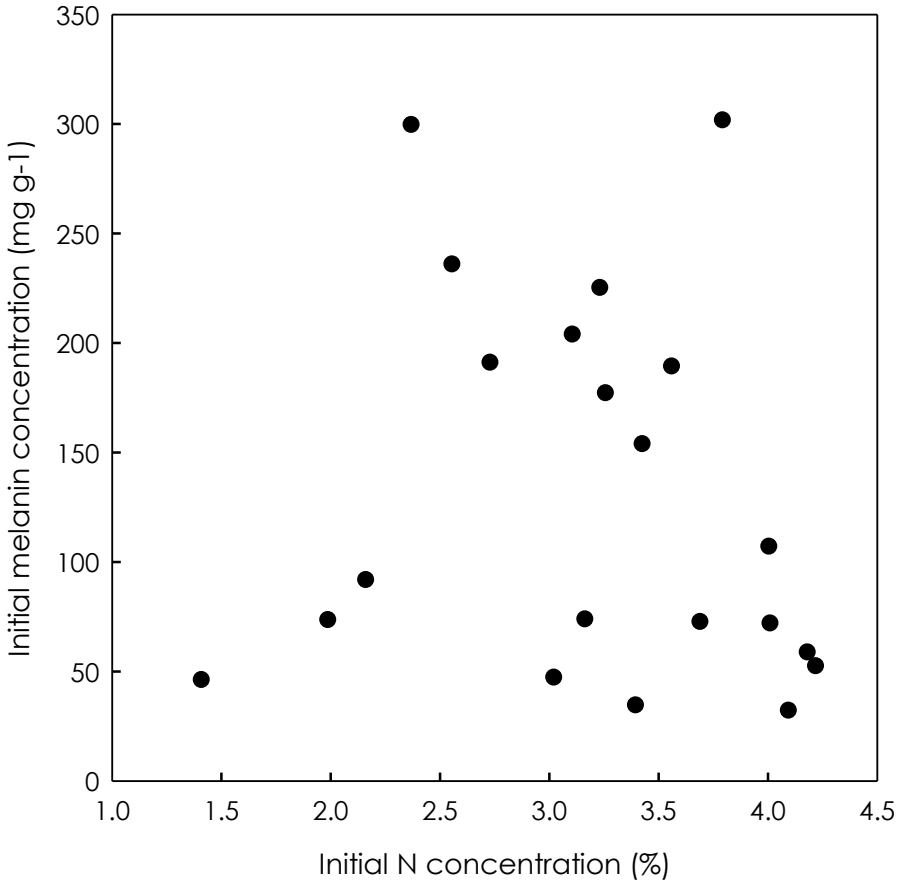
Source	**DF**	**SS**	**MS**	***F***	***P***	
Model	19	0.7953	0.0419	2.6696	0.0061	**
Error	34	0.5331	0.0157			
Corrected Total	53	1.3284				
Effect Tests						
Isolate	2	0.0386		1.6571	0.2019	
Treatment	1	0.2674		22.9560	<0.0001	***
Time	2	2.3778		68.0474	<0.0001	***
Isolate x Treatment	2	0.0394		1.6894	0.1959	
Isolate x Time	4	0.0590		0.8440	0.5427	
Treatment X Time	2	0.1044		2.9867	0.0407	*
Isolate x Treatment x Time	4	0.0396		0.5659	0.7552	

Figure 3.5. Mean percent mass remaining of necromass from three *Cenococcum geophilum* isolates (SC032, CgTor109, CeG02) that had been under normal conditions (closed circles) or with the melanin inhibitor (open circles) over the course of three months. Significant differences in mean decomposition between control and melanin inhibited necromass at each time point were determined with F-tests and are indicated with asterisks (P<0.001 (**); P<0.05 (*).



Supplemental Material

Figure 3.S1. Scatterplot of initial melanin and nitrogen (N) concentrations of the necromass samples showing no correlation between these predictors that were used in the multiple regression model in Experiment 1.



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CHAPTER 4

The function of melanin in the ectomycorrhizal fungus *Cenococcum geophilum* under water stress

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Abstract

Despite their ubiquity and importance to ecosystem function, our understanding of the functional ecology of ectomycorrhizal fungi remains poor. The highly melanized and common ectomycorrhizal fungus, *Cenococcum geophilum,* is drought tolerant and abundant in water stressed habitats, yet the responsible functional traits have not been identified. The production of melanin, a class of complex dark polymers found in fungal cell walls, may be a key functional trait to water stress tolerance. To test this hypothesis, we devised a series of experiments determining the effect of the melanin biosynthesis inhibitor, tricyclazole, on response to osmotic and desiccation stresses. Melanin inhibition only had negative effects on growth when *C. geophilum* isolates were subjected to water stress but not under control conditions. This suggests that melanin production is an important functional trait that contributes to water stress tolerance of this cosmopolitan ectomycorrhizal fungus.

Keywords

Cenococcum geophilum; ectomycorrhizal fungi; functional traits; melanin; water stress tolerance

Introduction

Ectomycorrhizal (EM) fungi are involved in symbiotic relationships with plant species in temperate, boreal and tropical forests (Smith & Read 2008) and are major drivers of ecosystem processes in these systems (Read and Perez-Moreno 2003). These fungi colonize fine roots and provide the tree with nutrients absorbed from the soil while they receive photosynthate. Ectomycorrhizal fungal communities are often extremely diverse (Walker *et al.* 2005; Tedersoo *et al.* 2006; Dickie 2007), yet our understanding of the functional diversity of these fungi, and how such diversity relates to biogeography and local abundance, is limited (Koide *et al.* 2011a).

The asexual Ascomycete, *Cenococcum geophilum*, is one of the most ubiquitous and abundant EM fungal species found in forest communities (Trappe 1962). While having a relatively wide niche breadth (Dickie 2007) this species (or complex of cryptic species) appears to be particularly well adapted to water-stressed conditions (Piggott 1982). The relative abundances of *C. geophilum* ectomycorrhizas and extramatrical mycelium have been shown to increase with water stress in the field (Pigott 1982; Querejeta *et al.* 2009). Tolerance to water stress has also been confirmed in pure culture studies using osmotically adjusted media (Mexal & Reid 1973; Coleman *et al.* 1989), cell damage assays following desiccation (diPeitro 2009), and respiration measurements under water stress (Jany *et al.* 2003). However, the physiological traits responsible for success of this species under water stress are poorly understood.

Tolerance of environmental stress is often accomplished through the employment of a suite of traits (Chapin *et al.* 1993). For example, the production and accumulation of compatible osmolytes (Jennings & Burke 1990; Bois *et al.* 2006), heat shock proteins (Kerner *et al.* 2012),

and hydrophobic proteins (Unestam & Sun 1995) have all been identified as traits that may be important in the tolerance of water stress in fungi. Another physiological trait that fungi, and *C. geophilum* in particular, may employ to tolerate drought stress is the production of melanin in their cell walls. Melanins are a group of complex polymeric compounds composed of indolic and phenolic monomers that are found in animals, bacteria, plants and fungi (Butler & Day 1998). The four types of melanin produced by fungi include 3,4-dihydroxyphenylalanine (DOPA), γ-glutaminyl-4-hydroxybenzene (GDHB), both of which are present in Basidiomycetes, and 1,8-dihydroxynaphthalene (DHN) and catechol melanin, which are produced primarily by Ascomycetes (Butler & Day 1998). These polymers are deposited in the cell walls of hyphae and spores (Bell & Wheeler 1986). The functional roles of these polymers in fungi are often *assumed* but less frequently experimentally tested. In the relatively few studies that have examined the role of these pigments there is general agreement in the ability of these polymers to ameliorate environmental stress, including UV radiation (Wang & Casadevall 1994), high temperature (Rosas & Casadevall 1997), and osmotic stress (Rehnstrom & Free 1996; Kogej *et al.* 2007; Kejžar *et al. In press*). Singaravelan *et al.* (2008) have shown higher melanin production in *Aspergillus niger* conidial spores along a natural stress gradient that include increased UV, temperature and water stress, which suggests that melanization is likely an important trait to the success of this species under these conditions.

Curiously, the high degree of melanization is often used to describe *C. geophilum,* yet the functional role of the dark polymer in this fungus has gone unexamined. Given the amount of resources that *C. geophilum* allocates to the production of melanin it is probable that it provides some benefit to the fungus. We hypothesize that melanin production in *C. geophilum* increases tolerance to osmotic stress and desiccation. To test these hypotheses, we inhibited melanin

biosynthesis of *C. geophilum* isolates and exposed them to osmotic stress and desiccation in a series of pure culture experiments.

Methods

Isolates, water potential optima, and melanin inhibition

Three isolates of *Cenococcum geophilum* were used to capture some of the genetic and phenotypic variation that is found in this species. These included SC032 (State College, PA, USA), CeG02 (Mt. Fuji, Japan), and CgTor109 (Southern Sweden). Prior to the experiments isolates were maintained on half strength potato dextrose agar (PDA; Difco).

In order to obtain the water potential optima for each of the isolates, four replicates of each isolate were grown at each of several water potentials including -0.01 (control), -0.3, -1.5, -2.0, and -3.0 MPa. This range was selected based on Coleman *et al.* (1989). Water potentials were adjusted using different concentrations of polyethelene glycol-6000 (PEG) (Aldrich #81260), an inert osmoticum that does not confound the effects of osmotic stress with toxic effects that are inherent with polyols or salts. Because PEG prevents the solidification of agar media, half strength potato dextrose broth (PDB; Difco) was used as the growth medium in these studies. Water potentials of the media were confirmed using a Wescor C-52 sample chamber and Wescor HR-33T microvoltmeter. To promote oxygen diffusion to the growing mycelium during this experiment, mycelium plugs (5 mm) from the agar cultures were placed on nitrocellulose membrane filters (Pall #66278), which were placed on absorbent pads (Pall #66190) containing 5 mL of PDB medium of the appropriate water potential. Photographs of the colonies were taken

at 7 day intervals using a Cannon Powershot ELPH 300HS. Colony area was determined by analyzing the images in ImageJ (Schneider *et al.* 2012). Relative growth rates were calculated by taking

$$RGR = \frac{\ln A\,1 - \ln A\,2}{t}$$

Where:

A1= initial colony area

A2= colony area at 21 days

t = number of days (21)

The first 21 days was used to calculate RGR because this was determined to be the exponential growth phase for all three isolates. RGR was analyzed using one-way analysis of variance.

The inhibition of DHN melanin production was achieved using the DHN melanin inhibitor tricyclazole (5-methyl-1,2,4-triazolo(3,4,b)-benzothiazole). Tricyclazole inhibits the key reductase enzymes in the DHN biosynthetic pathway (Butler & Day 1998). Concentrations of the inhibitor used in the melanin inhibition experiments were selected based on the response to tricyclazole by *Cenococcum geophilum* in preliminary studies. We found that up to 100 μg tricyclazole ml^{-1} of growth medium partially inhibited melanin biosynthesis and did not adversely affect the growth of *C. geophilum* relative to control under standard (unstressed) conditions. Tricyclazole is not highly water-soluble so it was dissolved in a small volume of ethanol (750 μL) before being added to the growth medium (125ml). All control treatments had equal additions of ethanol.

To examine the effects of the melanin inhibition on the cell wall structure of *C. geophilum* we took transmission electron micrographs of the isolates grown under control and melanin inhibited conditions. A buffered 2% glutaraldehyde fixative was used for primary fixation of the samples for 2 hours or longer. A secondary fixation was performed by placing samples in buffered 1% OsO_4 solution for 2 hours at room temperature. Samples were washed with buffer six times for 10 minutes after each fixation step. After fixation samples were stained with 0.5% uranyl acetate overnight at 4^0 C. Samples were then dehydrated using acetone through the following series 10%, 30%, 50%, 70%, 95%, and three times in 100% for 10 minutes each step. A 1:2 solution of resin : acetone was applied to the samples for 1 hr followed by a 2:1 solution resin : acetone was applied to the samples for 1 hr. Finally the samples were placed in 100% resin for 1 hour. The samples were then embedded and allowed to polymerize with 100% resin overnight in a polymerization oven. Transmission electron micrographs were taken with FEI Tecnai T-12 microscope. Cell wall thickness was determined by analyzing the micrographs in ImageJ (Schneider *et al.* 2012).

Experiment 1: Melanin and osmotic stress

To test the importance of melanin on growth of *C. geophilum* under reduced osmotic potential, a three factorial experimental design was used with isolate (3 levels), osmotic stress (2 levels), and melanin inhibition (2 levels) as the factors. For the osmotic stress the two levels used were unadjusted medium (-0.01 MPa) and PEG-adjusted medium (-1.7 MPa). This is roughly half the osmotic potential that most *C. geophilum* isolates tolerated in the study conducted by Coleman *et al.* (1989) and roughly the level of stress that moderately reduced the growth of the three isolates in our preliminary studies. Melanin inhibition comprised two levels: control and inhibited (66 μg tricyclazole ml^{-1} medium). Plugs of mycelium (5 mm diameter) were taken from each of the

isolates maintained on PDA and were then placed in PDB that was either untreated (control) or treated with the inhibitor (Fig. 4.S1). A total of 4 replicates were used per treatment combination (2 stress levels x 2 levels of melanin inhibition) for each of the 3 isolates (n= 48). Two plates in the experiment were subsequently removed from analysis because of contamination. The RGR was determined as above. A three-factor ANOVA was conducted with RGR as the response variable and isolate, osmotic stress, and melanin inhibition as the factors. The significance of mean differences was determined using Tukey's HSD test at $\alpha = 0.05$. Analyses were conducted using JMP Pro 10 (SAS Institute, Cary, NC, USA).

Experiment 2: Melanin and desiccation

To determine the role of melanin production in tolerating desiccation we took 5 mm diameter mycelia plugs of the isolates that had been grown on PDA, either with 100 μg tricyclazole ml^{-1} (melanin biosynthesis inhibited) or without inhibitor (inhibitor control). Both inhibited and control cultures were either transferred to new petri dishes containing half strength PDA (desiccation control) or completely desiccated over the course of 3 days (desiccated). In the desiccation treatment, plugs were placed in a sterile petri dish, which was placed in a desiccator with silica gel desiccant. After 3 days the desiccated mycelial plugs were placed on 5 mL of half-strength PDA in sterile petri dishes (n=60) (Fig. S1). As in Experiment 1, images were taken weekly for 8 weeks and colony areas were measured using ImageJ. All replicates were included in the analysis regardless of whether they survived the desiccation treatment. Isolate, melanin inhibition treatment, desiccation treatment, time and their interactions were analyzed with a repeated measures MANOVA test. Due to a significant four-way interaction the effects of

melanin inhibition treatment and time were examined individually for each isolate and desiccation treatment combination using one-way repeated measures MANOVA tests. All analyses were performed using JMP Pro 10 (SAS Institute, Cary, NC, USA).

Results

Water potential optima and melanin inhibition

There was some variation in the growth rates of CeG02 across the range of water potentials (Figure 4.1). There were no significant differences in RGR among any of the water potentials for isolates SC032 (one-way ANOVA; $F_{4,14}$= 1.257; P=0.333) and CgTor109 (one-way ANOVA; $F_{4,15}$=2.220; P=0.116). CeG02 was the most sensitive isolate to reduced water potentials showing significant reductions in RGR at water potentials lower than -1.5 MPa (one-way ANOVA; $F_{4,15}$=14.285; P<0.0001). Irrespective of their sensitivity to declining water potential, all three isolates were able to tolerate and grow at water potentials as low as -3.0 MPa. Tricyclazole treatment reduced the cell wall thickness of all three isolates (Table 4.1; Figure 4.1c,d) and changed the colony color of the isolates from jet black to a dark red-brown color (Figure 4.1 a,b).

Experiment 1: Melanin and osmotic stress

Consistent with the water potential optima results, there was variation among isolates in growth rate and in response to the reduction of osmotic potential across the three isolates (Table 4.2). The ANOVA model indicated that there was a significant interaction between osmotic stress and melanin inhibition (Table 4.2). Post-hoc analysis revealed that melanin inhibition by tricyclazole

did not significantly affect the RGR of the isolates under the control treatment (Figure 4.3) but it did cause a significant decline in the RGR of all the isolates of *C. geophilum* when the osmotic potential was -1.7 MPa (Figure 4.3).

Experiment 2: Melanin and desiccation

There was a significant four-way interaction between isolate, melanin inhibition treatment, desiccation treatment, and time in the full repeated measures MANOVA model ($P<0.0001$). The inhibition of melanin production did not negatively affect the growth in the cultures receiving no desiccation (Table 4.3; Figure 4.3a,b,c). In fact, there was faster growth in isolates SC032 and CgTor109 under melanin inhibition after the exponential growth phase when they received no desiccation. When melanin synthesis was not inhibited, all three isolates were able to tolerate the desiccation treatment and were able to recover and grow at rates similar to that exhibited by the controls. However, consistent with patterns found in Experiment 1, we found growth rate reductions among the cultures that were treated with tricyclazole when exposed to desiccation in all three isolates (Table 4.3; Figure 4.3d,e,f). Some mycelia plugs did not survive the desiccation treatment and, therefore, did not grow upon rehydration. This included SC032 n=1 (melanin inhibited); CeG02 n=2 (control); CeG02 n=8 (melanin inhibited)). The inhibitor had the least effect on response to desiccation for CgTor109, but even this isolate exhibited a significant decline in growth between 20 and 40 d due to melanin production inhibition (Figure 4.3e). Both SC032 and CeG02, on the other hand, were quite sensitive to desiccation when melanin production was inhibited (Figure 4.3d,f). CeG02 was, again, the most sensitive isolate to water

stress when melanin synthesis was inhibited, as the growth rates of cultures were either severely reduced or they did not recover at all from desiccation.

Discussion

Water stress tolerance of the isolates

The relatively high water stress tolerance of *C. geophilum* is well documented (Piggott *et al.* 1982; Coleman *et al.* 1989; Jany *et al.* 2003; diPietro *et al.* 2007). Thus, it comes as little surprise that the three isolates included in this study were able to tolerate and even thrive under water stress. Both CgTor109 and SC032 were able to tolerate low osmotic potentials and desiccation and saw no significant decline in RGR with the reduced water potential treatments, while CeG02 appeared to be more negatively affected by water stress, in terms of growth. There is precedent for some isolates of *C. geophilum* to be more tolerant of water stress than others (Coleman *et al.* 1989).

Melanin and water stress tolerance

In this study we show evidence consistent with the hypothesis that melanin production is an important functional trait in tolerating water stress in *C. geophilum*. The melanin biosynthesis inhibitor, tricyclazole, clearly reduced the production of melanin and affected the cell wall structure of the *C. geophilum* isolates. The inhibitor caused all isolates to have a lighter color (dark brown) than the normal jet-black characteristic of control cultures. Also, the hyphal cell wall thickness of inhibited cultures, as determined by transmission electron micrograph image analysis, was reduced compared to those of the hyphae of the inhibitor controls. These data are

consistent with the fact that melanin production in *C. geophilum* follows the DHN melanin biosynthesis pathway.

Under normal (unstressed) conditions, melanin inhibition did not negatively affect the growth rate of any of the isolates. However, when grown under osmotic stress, there were significant reductions in growth rate in all three of the isolates when melanin production was inhibited. While the osmotic potential of growth media adjusted with a non-toxic osmoticum, such as PEG, is a good approximation of soil water potential (Verslues *et al.* 2006), PEG cannot be easily used to approximate the extreme reduction in water potential caused by desiccation. However, periods during which microbes experience extremely low water potentials, those that force microbes to go dormant or die, are common (Schimel *et al.* 2007). Thus, in addition to the osmotic stress experiment, we performed an experiment to test the role of melanin in the tolerance of desiccation by *C. geophilum* isolates. As in Experiment 1, inhibition of melanin synthesis did not negatively affect the growth rate of the isolates under control conditions. In fact, both SC032 and CgTor109 had faster growth rates after the exponential growth phase, which may be related to additional energy allocated to growth as a consequence of reduced melanin production. We found a significant reduction in recovery from desiccation in all of the isolates when melanin production had been inhibited. Together these results suggest that the melanization of hyphal cell walls in *C. geophilum* is an important functional trait in the tolerance of osmotic stress and desiccation.

Cell wall melanization may have beneficial effects as water stress is imposed. Some have noted that the porosity of cell walls is decreased by melanin (Howard *et al.* 1991). Reduced porosity may slow the rate of water loss from the cell, at least initially, upon reduction in soil osmotic potential or water content. Moreover, the hyphae produced by *C. geophilum* have been

noted to be strongly hydrophilic (Unestam & Sun 1995) which may be a function of cell wall melanization (Butler & Day 1998). Hydrophilic hyphae may allow fungi to hold onto water when water availability becomes limiting.

The maintenance of turgor pressure is important to fungal cell growth (Bartnicki-Garcia *et al.* 2000), which is achieved through the production or accumulation of compatible solutes (Jennings & Burke 1990; Bois *et al.* 2006). So-called osmotic adjustment increases as water stress is imposed (Money *et al.* 1998) in order to maintain turgor in spite of declining water potentials. There is evidence that *C. geophilum* accumulates high concentrations of compatible solutes relative to other ectomycorrhizal fungi (Koide *et al.* 2000). It has been suggested that melanized cell walls may help retain these molecules from leaving the cell, increasing the success of the fungus under water stress (Kogej *et al.* 2007). Moreover, following a period of osmotic adjustment due to water stress, sudden rewetting may result in very high turgor pressures, creating enough strain on the cell wall to cause cell lysis. Melanization may strengthen the cell wall, allowing the cell to withstand elevated turgor pressure resulting from rewetting following water stress.

Lastly, water stress can lead to the production of free radicals, which are harmful to cell function (Kranner & Birtić 2005). Melanin has the ability to scavenge and neutralize oxidants (Jacobson *et al.* 1995), which may be of particular advantage during water stress.

Implications

Various researchers have suggested that melanin increase tolerance to several environmental stresses including freezing (Robinson 2001), metal toxicity (Gadd & de Rome 1988) and

hypersalinity (Kogej *et al.* 2007; Kejzar *et al. In press*), all stresses to which *C. geophilum* has been shown to be tolerant (Saleh-Rastin 1976; Corbery & Le Tacon 1997; Gonçalves *et al.* 2009). We show here that melanin may also function to increase tolerance to water stress, including both osmotic stress and desiccation. Thus, melanin production may be a key functional trait that allows fungus *C. geophilum* to have a wide niche breadth (Dickie 2007) and a global distribution. While *C. geophilum* is one of the most frequent and conspicuously melanized EM fungi, melanin production may be utilized by other EM fungi and other root associated fungi to tolerate water stress. However, it is unclear whether Basidiomycotan melanins, which have a different biosynthetic pathway, have similar drought protective properties.

Ectomycorrhizal fungi represent a significantly large litter input in forest ecosystems (Cairney 2012; Clemmensen *et al.* 2013; Ekblad *et al.* 2013). Ectomycorrhizal fungi also affect fine roots, another large litter input (Langley *et al.* 2006; Koide *et al.* 2011b). It appears that most fungal necromass is decomposed relatively quickly (Koide & Malcolm 2009; Drigo *et al.* 2012; Fernandez & Koide 2012). However, fungi that are heavily melanized appear to be relatively recalcitrant to decomposition (Fernandez & Koide Chapter 3). Fernandez *et al.* (2013) have found that the ectomycorrhizas of this species had persistence times that were 4-10 times longer than other morphotypes. Thus, melanized EM fungi such as *C. geophilum*, may disproportionately influence the stability of this important litter pool, potentially increasing the C and nutrient sequestration where it is abundant.

Conclusion

In this series of experiments we showed the importance of melanin production to water stress tolerance in *C. geophilum*. This likely stems from its effects on cell walls including a reduction in cell wall porosity, an enhanced hydrophilicity and increased strength, permitting *C. geophilum* to retain more water during mild water stress, and resist lysis during rewetting following desiccation. Melanization may be a key trait allowing this species to tolerate seasonal water stress as well as chronic aridity. We speculate further that this trait contributes to the relatively wide niche of *C. geophilum.*

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Figures and Tables

Table 4.1. Mean cell wall thickness (± SE) of *Cenococcum geophilum* isolates untreated and treated with the melanin inhibitor, tricyclazole, assessed from cross sections of hyphae captured by transmission electron micrographs (*n*= 85).

	Cell wall thickness (µm)(±SE)	
Isolate	**Control**	**Melanin inhibited**
SC032	0.299 (0.014)	0.135 (0.012)
CgTor109	0.345 (0.026)	0.285 (0.015)
CeG02	0.533 (0.048)	0.324 (0.019)

Figure 4.1. Photographs of *Cenococcum geophilum* isolate CeG02 growing on PDA either untreated (A) or treated with 100 µg tricyclazole ml^{-1} (B). Transmission electron micrographs of cross-sections of hyphae from the *Cenococcum geophilum* isolate CeG02 control (C) and treated with 100 µg tricyclazole ml^{-1} of the melanin synthesis inhibitor tricyclazole (D). The cell walls are indicated by CW.

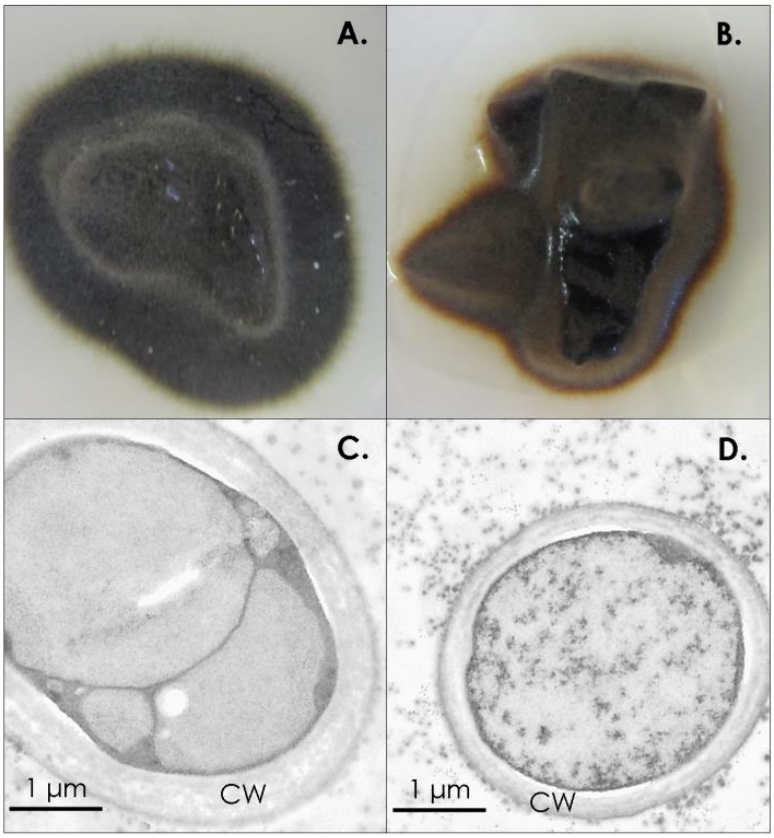


Figure 4.2. Mean relative growth rates (RGR) ± SE at various osmotic potentials for the three *Cenococcum geophilum* isolates.

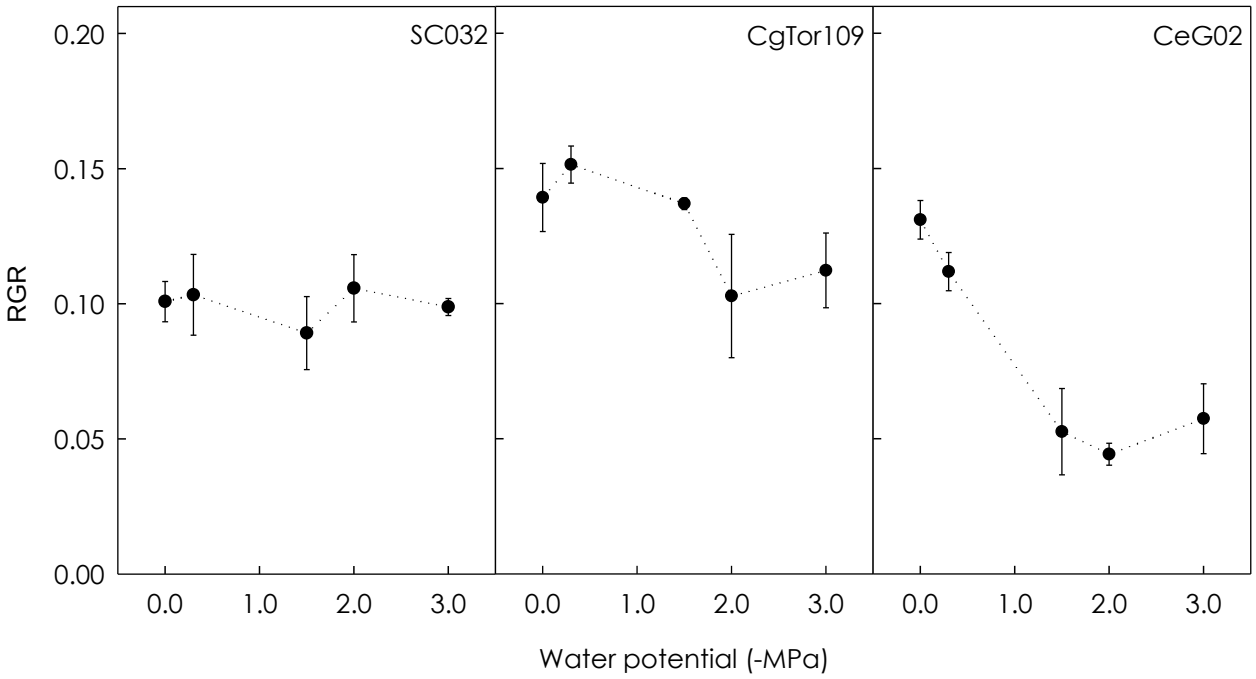


Table 4.2. Results of the three-way ANOVA tests for relative growth rate with isolate, drought treatment, melanin inhibitor as explanatory variables in Experiment 1.

Source of variation	**DF**	**SS**	**MS**	***F* Ratio**	***P***	
Model	11	0.04695	0.00427	15.297	<0.0001	*
Error	31	0.00865	0.00028			
Corrected total	42	0.0556				
Effect Tests						
Isolate	2	0.02175		38.972	<0.0001	*
Osmotic Stress	1	0.00593		21.269	<0.0001	*
Inhibitor	1	0.00342		12.265	0.0014	*
Isolate X Osmotic Stress	2	0.00495		8.875	0.0009	*
Isolate X Inhibitor	2	0.00125		2.239	0.1235	
Osmotic Stress X Inhibitor	1	0.00325		11.658	0.0018	*
Isolate X Osmotic Stress X Inhibitor	2	0.00089		1.591	0.2199	

Figure 4.3 Results from Experiment 1. Mean relative growth rates (RGR) ± SE. Dark bars indicate control water potential treatments while light gray indicates the reduced water potential (-1.7 MPa) using the osmoticum polyethylene glycol. Diagonal patterned bars indicate melanin inhibition (MI, 66 μg tricyclazole ml^{-1})

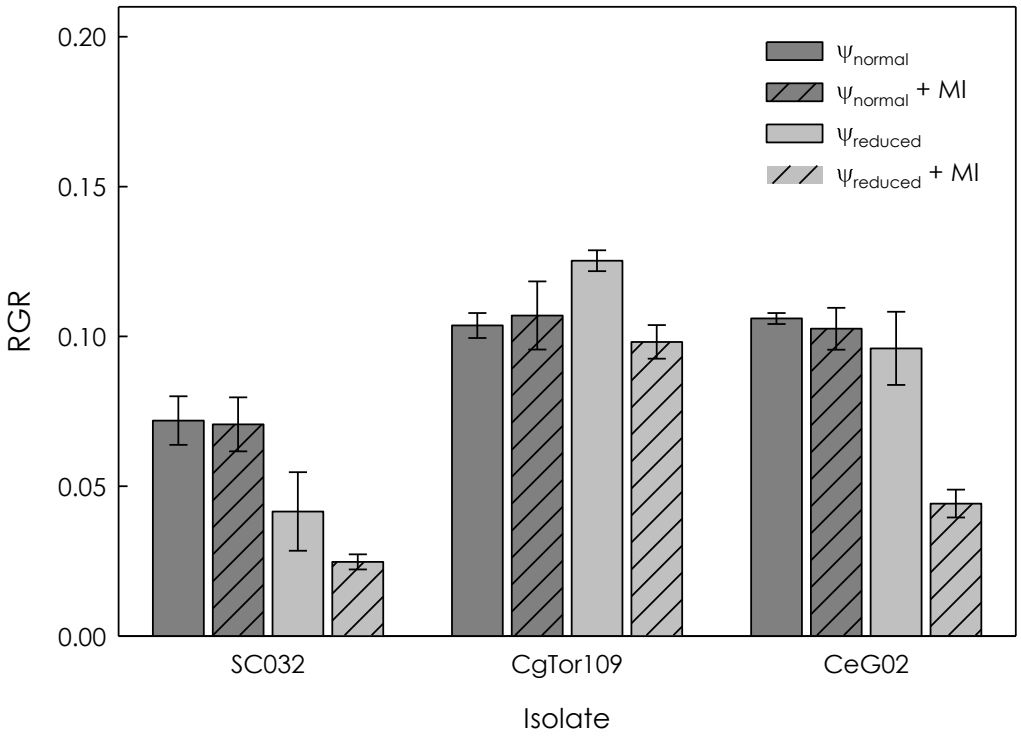
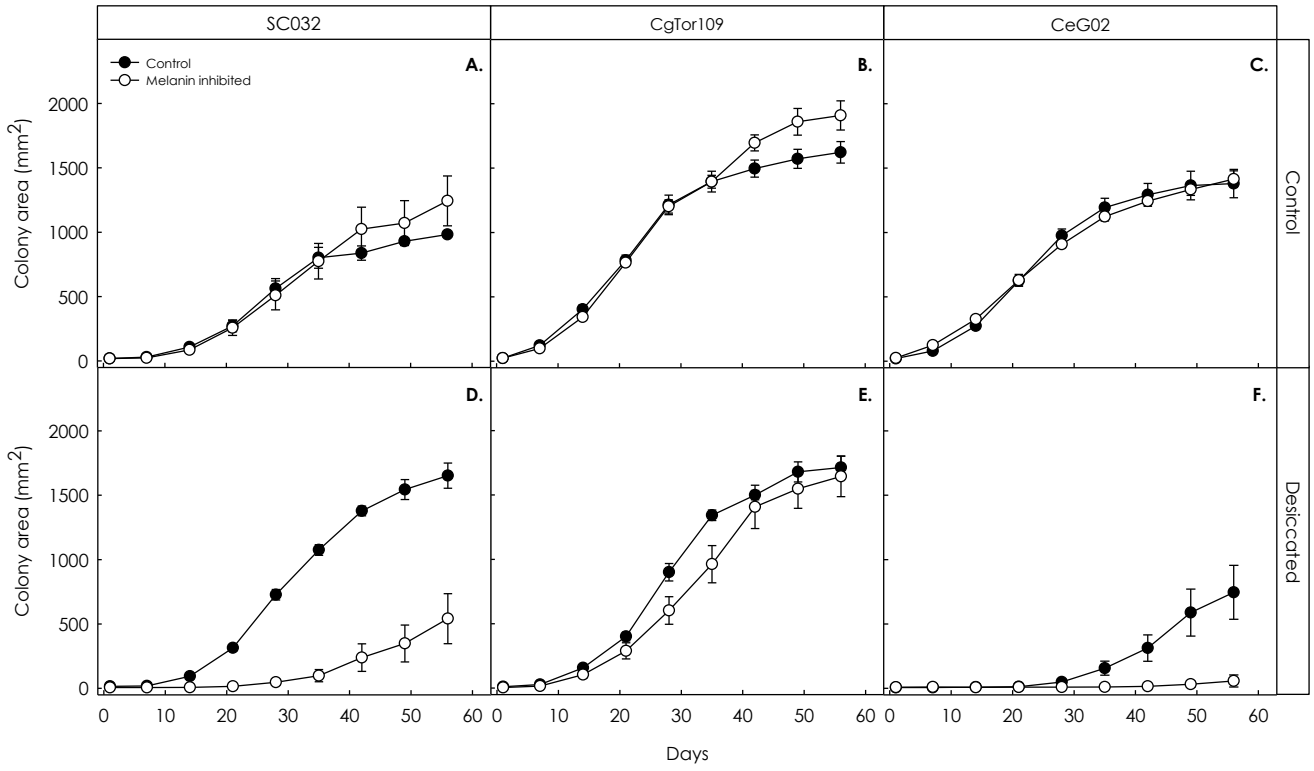


Table 4.3. Results of the repeated-measures MANOVA tests for colony area of non-desiccated and desiccated mycelia of the three isolates with inhibitor and time as explanatory variables.

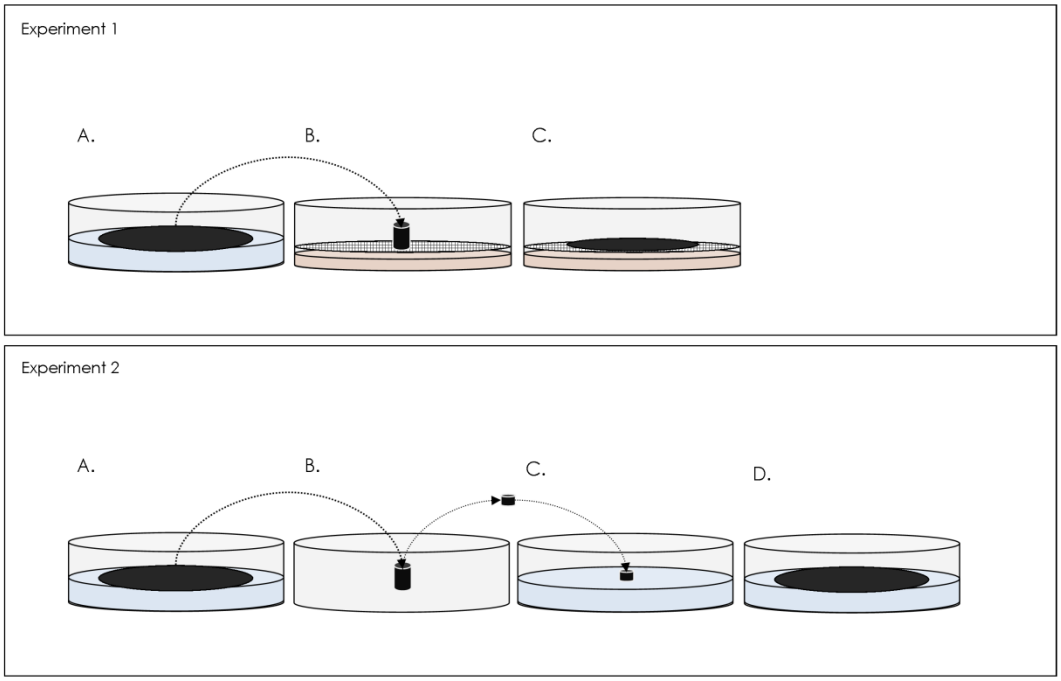
Source		Value	Exact F	NumDF	DenDF	*P*	
Control							
SC032	Inhibitor	0.023106	0.2773	1	12	0.6081	
	Time X Inhibitor	13.52144	8.4509	8	5	0.0155	*
CgTor109	Inhibitor	0.108135	1.2976	1	12	0.2769	
	Time X Inhibitor	16.91842	10.574	8	5	0.0094	*
CeG02	Inhibitor	0.002252	0.027	1	12	0.8722	
	Time X Inhibitor	5.24825	3.2802	8	5	0.1032	
Desiccation							
SC032	Inhibitor	5.358259	96.4487	1	18	<0.0001	*
	Time X Inhibitor	23.40515	32.1821	8	11	<0.0001	*
CgTor109	Inhibitor	0.189835	2.8475	1	15	0.1122	
	Time X Inhibitor	16.70926	16.7093	8	8	0.0003	*
CeG02	Inhibitor	0.845496	11.8369	1	14	0.0040	*
	Time X Inhibitor	4.678839	4.094	8	7	0.0396	*

Figure 4.4. Results from Experiment 2. Mean colony area ± SE over time for the three *Cenococcum geophilum* isolates (SC032 A & D; CgTor109 B & E; CeG02 C & F) in the control (A-C) and desiccation treatments (D-F). Open circles represent mycelia grown with the melanin inhibitor (66 μg tricyclazole ml^{-1}) and the closed circles represent the controls



Supplemental Material

Figure 4.S1. Diagram showing the processes of the two experiments. **Experiment 1**. **A.** 5mm plugs of mycelium were taken from *C. geophilum* pure-culture isolates maintained on potato dextrose agar (PDA) and **B.** transferred to the two factorial experimental petri dishes containing the pads and membrane filters used to suspend the mycelia in potato dextrose broth (PDB) that had been amended with polyethylene glycol 6000 (osmotic stress treatment) and tricyclazole (Melanin inhibition treatment) or controls. **C.** Colony areas were measured weekly and relative growth rates determined. **Experiment 2. A.** 5 mm plugs of mycelium were taken from *C. geophilum* pure culture isolates maintained on potato dextrose agar (PDA) which had been either amended with tricyclazole (melanin inhibition) or unammended. **B.** Plugs were then completely desiccated for 3 days in sterile petri dishes **C.** Desiccated plugs were then placed on normal PDA and allowed to rehydrate and recover from desiccation. **D.** Colony areas were measured weekly and relative growth rates determined



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Chapter 5

Towards a better understanding of the factors influencing the decomposition dynamics of ectomycorrhizal fungal litters

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Abstract

The turnover of ectomycorrhizal fungal biomass represents a significant input into forest carbon (C) and nutrient cycles. Given the size of these fluxes, understanding what factors control the decomposition dynamics of these litters has become an increasingly important line of research in the hope of a better understanding of soil organic matter formation and nutrient cycling in ecosystems. Recent research has highlighted the considerable variation in the decomposition rates of EM fungal litters and patterns from this research are beginning to emerge. In this article we review the research that has examined both intrinsic and extrinsic factors that control the decomposition of these litters and propose additional factors that are likely to have great influence on decomposition dynamics and influence ecosystem properties.

Keywords

carbon; chitin; decomposition; ecosystem function; ectomycorrhizal fungi; melanin; soil organic matter; nutrient cycling

1. Introduction

Ectomycorrhizal (EM) fungi play a critical role in carbon and nutrient cycling in ecosystems. The influence EM fungi on nutrient uptake has been well documented and its implications for ecosystem processes are now well appreciated (Read 1991; Read & Perez-Moreno 2003; Courty *et al.* 2010; Orwin *et al.* 2011). In addition, we are beginning to recognize the significant contributions EM fungi make to forest carbon (C) and nutrient cycles as result of the necromass produced from the death of their tissues (Fogel & Hunt 1983; Treseder & Allen 2000; Langley & Hungate 2003; Godbold *et al.* 2006; Cairney 2012; Clemmenssen *et al.* 2013; Ekblad *et al.* 2013). Until recently not much attention has been paid to EM fungal litter inputs. This is largely due to the fact microbial litter inputs have been considered to be relatively insignificant because standing pools of microbial biomass are relatively small compared to those of standing plant biomass. In reality, large quantities of C and nutrients flow through the microbial pool, making its contribution to soil organic matter (SOM) large (Grandy & Neff 2008). Mycorrhizal fungi are unique among fungi in that the majority of their C is allocated to them by host plants (Hobbie *et al.* 2002) and, in terms of their C source, are analogous to fine roots. The amount of C that is allocated to these fungi has been estimated to range from 1-20 % of annual net primary productivity (Hobbie 2006). Consequently the turnover of this biomass results in a large litter input into C and nutrient cycles (Godbold *et al.* 2006). In addition, the intimate association of the EM fungi with fine roots alters their biochemistry and decomposition, which is another very large litter input (Langley & Hungate 2003). Recently, Clemmensen *et al.* (2013) have provided evidence which suggests that a very large portion of SOM in a boreal forest system was of fungal and root origin, supporting the hypothesis that belowground litter

inputs are just as significant, if not more significant, than aboveground litter inputs to SOM formation in forest ecosystems.

Given the large fluxes of C and nutrients entering the soil through these litters and their disproportionate contribution to SOM, understanding the decomposition dynamics of these litter inputs has become an increasingly important line of research (Figure 5.1.). In this work we focus on reviewing research focusing on the controls on the decomposition of ectomycorrhizal fungal litter and the implications to biogeochemical cycles. We also present discuss future directions within this growing research area.

2. Litter quality and biochemistry of ectomycorrhizal fungal litters

2.1 Carbon and nitrogen concentrations in fungal litter

Litter quality is a major control on the decomposition of litter (Melillo *et al.* 1982; Berg 1984). Quality is determined by both the concentration of growth-limiting nutrients (i.e. C, N, P) and the recalcitrance, or the resistance to decomposition, of the molecules comprising the litter. The growth of microbial decomposers is primarily limited by C but are also be limited by N (Schimel & Weintraub 2003). Decomposition is generally favored by C:N ratios lower than which are found in plant litter (Cleveland & Liptzin 2007), and which helps maintain the C:N stoichiometry in microbial cells (Manzoni *et al.* 2010; Sinsabaugh *et al.* 2010).

Litters with high concentrations of recalcitrant compounds tend to have slower decomposition rates due to their resistance to enzymatic breakdown (Melillo *et al.* 1982). The resistance to enzymatic breakdown is directly related to the energy required to break chemical bonds in the molecules as well as the lack of stereospecific binding sites required for hydrolytic

enzymatic reaction. Lignin is a compound found in plant tissues that is highly resistant to decomposition due to its structure. Its complex and irregular structure requires the implementation of extracellular oxidative enzymes by decomposers (Kirk & Farrell 1987). In plant litters, C:N and lignin:N ratios are typically good predictors of aboveground litter decomposition rates (Mellilo *et al.* 1982) and root litter (Berg 1984; Silver & Miaya 2001).

It is not unreasonable to expect the litter quality to control, at least in part, the decomposition rates of fungal litter. Koide & Malcolm (2009) tested the role of C and N contents on the decomposition rate of ectomycorrhizal fungal necromass in a litter bag study and found that initial N concentration was a good predictor of the decomposition rate of these tissues. Wilkinson *et al.* (2011) examined CO_2 efflux from soil microcosms amended with the EM fungal necromass and found no relationship between the C and N contents and the C:N ratio of the necromass and CO_2 emitted during decomposition. Inconsistencies between these studies may be the result of differences between *in situ* and *in vitro* approaches. For instance, the decomposer communities used in microcosm experiments may exclude key functional groups that are found in natural conditions and may result in different decomposition dynamics (i.e. live ectomycorrhizal fungi). Additionally, there may be other key biochemical factors influencing the quality of the fungal litter, much like lignin concentrations do in plants. In many cases lignin:N ratios are often better predictors of decomposition rates in plant tissues than C:N ratios are (Melillo *et al.* 1982). This is the result of the considerable variation in carbon quality of these tissues. While C and N concentrations are likely to influence decomposition to a certain degree because of the stoichiometric constraints of decomposer organisms, other biochemical factors will likely also have an influence on the decomposability of EM litters. Biochemistry varies

widely across fungal species and likely contributes to the wide variation we see in decomposition dynamics of ectomycorrhizal litters (Table 5.1.).

The cytoplasmic fraction likely plays a minor role in the decomposition of EM litters for two reasons. First, it is not clear that a significant portion of the cytoplasm remains in dying fungal tissue because fungi are able to move cytoplasm from one region of the mycelium to another (Saltarelli *et al.* 1998). Second, as with plant litters, this cytoplasmic fraction is highly labile and appears to be rapidly taken up and incorporated into the decomposer community. Therefore, it seems likely that the nature of the cell wall fraction exerts the most control on the decomposition of EM fungal litters.

2.2 Fungal cytoplasm

Common soluble compounds of the cytoplasm include sugars, lipids, polyols, amino acids, nucleic acids and various osmolytes. These compounds are released into the soil upon death and lysis of the fungal cell. As with plant litters, we would expect these compounds to be taken up and utilized by soil microbes very quickly because of their relatively simple chemical structure. Indeed, Nakas & Klein (1979) found that carbon from the cytoplasmic fraction of fungal cells was rapidly assimilated and respired, relative to C in the cell wall fraction.

Glycogen is an insoluble multibranched polysaccharide composed of glucose monomers linked with α- (1,4) and α- (1,6) glycosidic bonds and is utilized by fungi for energy storage. Glycogen concentrations in fungi ranges from 5% to 10% of total dry weight (Kalač 2009). The lability of glycogen is very high. Drigo *et al.* (2012) found that carbon from glycogen was rapidly degraded and incorporated into microbial decomposer biomass.

Lipids are typically rapidly decomposed and taken up by soil microbes (Moucawi *et al.* 1981) but this is not always the case. Ergosterol is a lipid unique to fungal cell membranes used in estimating biomass of fungi (Martin *et al.* 1990). It is assumed to be a good indicator of biomass because it is quickly degraded after cell death (Martin *et al.* 1990). This however has been challenged by Zhao *et al.* (2005) who found that ergosterol was not rapidly mineralized when added to microcosms and remained fungal tissue for weeks after death.

As hyphae senesce they may undergo vacuolization as the cytoplasm moves from one part of the mycelium to other parts through septal pores (Saltarelli *et al.* 1998). Vacuolization may result in lower quality litter. However, under certain circumstances the cytoplasmic may interact with the more recalcitrant components of the cell wall during decomposition. For example, if hyphae are suddenly disconnected from other parts of the mycelium, as through the activities of soil arthropods, then the presence of cytoplasm in the hyphae may prime the decomposition of the cell walls.

2.3 Cell wall fraction

A large portion of fungal cell mass is found in the cell wall (Ruiz-Herrera 1992). Once viewed as a static structure, the cell wall is now known to be a highly dynamic structure with its composition varying depending on factors such as age, genotype, taxa, and environmental conditions (Bartnicki-Garcia 1968; Wessels 1994; Bowman *et al.* 2006; Feofilova 2010). EM fungi, which are primarily members of the Basidiomycota and Ascomycota, have cell walls that are composed of β-glucans, chitin, glycoproteins, melanins and other minor components (Wessels 1994; Bowman 2006; Feofilova 2010). There appears to be wide variation in the

decomposition rates of fungal cell wall fractions across species (Hurst & Wagner 1969), which is probably related to variation in cell wall chemistry.

Polysaccharides: Glucans and chitin

The majority of the cell wall is composed of polysaccharides, which accounts for approximately 80-90% of the dry mass for most species (Bartnicki-Garcia 1968). The cell walls of Basidiomycete and Ascomycete fungi are anchored with carbohydrate microfibrils, composed of cross linked β-(1-3), β-(1,4) and β-(1,6)-glucans, as well as chitin polysaccharides suspended in a matrix composed of various glycoproteins and amorphous α-(1,3)-glucans (Feofilova 2010). Together, β-glucans and chitin are cross-linked with covalent bonds to form the polysaccharide microfibrils of the cell wall (Feofilova 2010).

Glucans are polysaccharides composed of glucose monomers bound with either an α- or a β- bond at different carbon units on the glucose monomer. Though glucans are major components of fungal cell walls, the role they play in decomposition of EM fungal litters is not clear. However, because they are structurally similar to cellulose, and because similar extracellular enzymes are employed by decomposers to depolymerize them, it is reasonable to expect that their decomposition dynamics would be similar. Additionally, some β-glucans can increase the water holding capacity of the cell wall (Kyanko *et al.* 2013), which may indirectly influence decomposition.

Chitin is one of the most common polymers found on earth, with estimates of hundreds of millions of tons produced by fungi and arthropods annually (Gooday 1990). Chitin is composed of n-acetylglucosamine mononmers with β-(1,4) linkages and, therefore, contains N.

Concentrations of chitin have been found as high as 20-30 % of dry weight in some filamentous fungi (Bowman & Free 2006) but the concentrations in EM fungi typically range from 1-10% of dry weight (Markkola *et al.* 1995; Ekblad *et al.* 1998; Fernandez & Koide 2012).

In recent years there has been some confusion surrounding the decomposition of the polysaccharide fraction of fungal cell walls, particularly chitin. Early work examining the decomposition of pure chitin in soil microcosms showed that pure chitin is decomposed more rapidly than pure cellulose when added to soil (Trofymow *et al.* 1983). However, more recently ecologists have made claims suggesting that chitin is a recalcitrant polymer in fungal litter and may result in a large contribution of fungal necromass to soil organic matter (SOM) (Treseder & Allen 2000). One of the problems with decomposition studies is that recalcitrance is not explicitly defined in many cases (see Schmidt *et al.* 2011). Fernandez & Koide (2012) explicitly examined the recalcitrance of chitin relative to all other fungal cell components by measuring changes in chitin concentration over the course of decomposition of ectomycorrhizal fungal necromass. For all the EM fungi isolates that were tested, a rapid decline in chitin concentration was found, suggesting that chitin was preferentially decomposed relative to other cell wall components. In addition, initial chitin concentrations were positively related to percent decomposition of the necromass. Supporting these findings, Drigo *et al.* (2012), used a microcosm experiment designed to examine the decay of cell wall components of the ectomycorrhizal fungus *Pisolitus microcarpus* utilizing stable isotope probing methods and found a rapid decline (within 10 days from addition) in the chemical functional groups associated with the glucan-chitin complex. Finally, Zeglin *et al.* (2013) found rapid assimilation of pure chitin and N-acetylglucosamine monomers when added to soil microcosms containing fungal-mat communities. Both fungi and bacteria produce a variety of extracellular enzymes, classified

as chitinases, that hydrolyze the polysaccharide's glycosidic bonds into simpler molecules making them available for uptake (Gray & Baxby 1968). Because chitin is a N-rich compound, its rapid degradation and incorporation may be driven by N limitations of decomposer fungi and bacteria that common in temperate forest soils. Taken together, this suggests that chitin, itself, is not resistant to decomposition relative to other compounds in fungal litter and may be an important source of both C and N to soil decomposer communities.

Protein

While only a minor component relative to polysaccharide, the proteins found in fungal cell walls vary greatly in function, chemistry, and potentially influence on decomposition dynamics of EM litter. Proteins are found in fungal cells and concentration can vary widely across fungal species (Christias *et al.* 1976). Approximately 60-70% of N found in fungal cell walls is found as protein (Smiderle *et al.* 2012).

Glycoproteins are another component of fungal cell walls. They have both structural and signaling functions (Bowman *et al.* 2006). Glomalin, a class of glycoprotein produced by arbuscular mycorrhizal (AM) fungi, has been shown to be relatively resistant to decomposition (Steinberg & Rillig 2003) and may be involved in the stabilization of soil aggregates (Rillig 2004). Glomalin may be resistant to decomposition because of its hydrophobic nature (Rillig & Mummey 2006).

It is unclear if EM fungi synthesize similar glycoproteins that are resistant to degradation, but hydrophobins (cystine rich hydrophobic proteins) are expressed outside the cell walls of Basidiomycete and Ascomycete hyphae (Wessels 1996; Wösten 2000; Rillig *et al.* 2007). These proteins are arranged as a film on the outside of the cell wall, making it unwettable (Wösten

2000). These proteins are important in the formation of ectomycorrhizas as well as playing a role in the retention and transportation of water in the extramatrical mycelium (Unestam & Sun 1995). The unwettable nature of these tissues is likely to influence their enzymatic decomposition (Rillig *et al.* 2007).

Melanin and other secondary metabolites

Melanins are complex, dark biopolymers that are produced by animals, bacteria and fungi. While the chemical structure of these compounds is poorly understood, they are composed of indolic and phenolic monomers (Wheeler & Bell 1986; Butler & Day 1998a). Fungi produce four classes of melanin that vary in their precursors and biosynthetic pathways. These include γ-glutaminyl-3,4-dihydroxybenzene (GDHB) melanin, dihydroxyphenylalanine (DOPA) melanin, dihydroxynaphthalene (DHN) melanin and catechol melanin (Butler & Day 1998a). Melanin type appears to be largely determined by phylogeny; Basidiomycetes produce GDHB and DOPA melanins while Ascomycetes produce primarily DHN melanin but may also produce DOPA and catechol melanins (Bell & Wheeler 1986; Butler & Day 1998a). Melanin content can vary tremendously across taxa with some fungi being completely hyaline and others heavily pigmented (Hurst & Wagner 1969; Butler & Day 1998a). Like lignin, melanins lack stereo-specific binding sites, which enzymes can target (Butler & Day 1998b). Two early studies using ^{14}C labeled fungal material amendments to soil microcosms examined the potential recalcitrance of melanin. Hurst & Wagner (1969) contrasted the mineralization of cell wall amendments from melanized and hyaline fungi and found that the cell walls from melanized fungi were mineralized relatively slowly when compared to those from hyaline fungi, which were rapidly mineralized.

These findings were later supported in a study by Malik & Haider (1982) who found that melanin fractions of fungal cells were mineralized at a slower rate than total cell wall and cytoplasm fractions in all fungal isolates tested. *Cenococcum geophilum* is one of the most abundant and ubiquitous EM fungal species globally, that also happens to produce a large amount of melanin in its hyphal cell walls (Pigott 1982). Fernandez *et al.* (2013) demonstrated with minirhizotron imaging and a vital stain technique that the ectomycorrhizas of *C. geophilum* are far more persistent in soil when compared to ectomycorrhizas produced by other species. The persistence of *C. geophilum* ectomycorrhizas were 4-10 times greater than ectomycorrhizas of other species suggesting that their decomposition was drastically reduced. Fernandez & Koide (Chapter 3) used a comparative approach and measured the decomposition rates of EM fungal necromass that varied in melanin concentration. They found that there was a significant negative relationship between melanin concentration and decomposition rate. This finding was strengthened with a second manipulative experiment where melanin biosynthesis of *C. geophilum* isolates was inhibited with the commonly used melanin inhibitor, tricyclazole. Again, there was a negative relationship between melanin concentration and decomposition rate. Together these studies show that melanin itself is clearly resistant to decomposition and reduces the overall decomposability of the fungal tissues in which it is found. This recalcitrance is likely a result of the complex aromatic nature of these polymers that require oxidative enzymes to degrade. While Butler & Day (1998b) found that peroxidases produced by a lignin decomposer fungus effectively degraded fungal melanin, melanin has been shown to inhibit common enzymes used in the process of other cell wall components including chitin and β-glucan (Kuo & Alexander 1967; Bull 1970). Thus, melanin may influence the overall decomposability of the fungal litter by effecting the decomposition of other components. Because of its recalcitrance, its

requirement for oxidative enzymes for degradation, and the large variation in concentrations across fungal species, melanin should be considered a fungal analogue to lignin in terms of biochemical control on the decomposition of fungal litter.

Fungi produce a myriad of secondary compounds that likely influence the decomposition of their tissues (Keller *et al.* 2005). These compounds can range widely in function but a large proportion appear to be involved in inhibiting the growth of bacteria and other fungi (Keller *et al.* 2005). A major area of research in mycorrhiza ecology is the protective role of EM fungi from root pathogens (Marx 1972; Fitter & Garbaye 1994). EM fungi have been shown in numerous studies to ward off fungal and bacterial root pathogens using secondary compounds. Garrido *et al.* (1982) surveyed the antimicrobial properties of 36 fungi in the Agaricales (both ectomycorrhizal and saprotrophic) and found that the extracts of the vast majority of these isolates inhibited the growth of one or more bacterial strains. Antibiotic activity has also been found in isolates of *C. geophilum* too (Krywolap & Casida 1964). In addition to antibiotic compounds, fungi also produce a wide array of volatile organic compounds (VOCs) that can have negative effects on competing fungi. Common EM fungal taxa often produce these compounds in large quantities (Krupa & Fries 1971) and may reduce the effectiveness of decomposer organisms by directly inhibiting growth near the EM fungal mycelium. The most well-known example of this is phenomenon occurs in soil where truffle producing EM fungi in the genus *Tuber* are dominant. These fungi produce large quantities of VOCs that effectively reduce the surrounding plant diversity by creating bare soil patches known as *brûlés* (French for "burnt"). The VOCs produced by *Tuber* sp. also drastically influence the microbial communities in these soils. Napoli *et al.* (2009) showed that fungal communities within brûlé soil were dominated by *Tuber melanosporum* (low species evenness) and had significantly lower species

richness compared to soil outside of brûlés. Basidiomycetes, in particular, declined. This drastic influence on fungal communities undoubtedly influences the biogeochemical cycling in these soils and may negatively impact litter decomposition. The residence time of these compounds in soil is probably an important factor in determining how much influence they have on decomposition dynamics of EM fungal litters. However, given the clumped distribution of some EM fungi (Lilleskov *et al.* 2006; Pickles *et al.* 2010) it is not hard to imagine patches of living mycelium producing antibiotic compounds or VOCs that negatively affect the decomposition of adjacent EM litters by making the surrounding soil toxic to decomposers. Ultimately, the effects of these compounds have on the decomposition dynamics of EM fungi have yet to be explicitly examined.

Some EM fungi produce copious amounts of oxalic acid, which reduces the pH of the surrounding soil and chelates calcium ions releasing nutrients such as phosphorous and sulfur available for absorption. As a result these fungi accumulate a great deal of oxalate crystals on and around their hyphae, which inhibits decomposition (Cromack et al 1977). Because of the proximity of these crystals to the hyphae, they may slow the decomposition of litters with heavy deposition of oxalate. Some bacteria and soil fauna may specialize on the consumption of oxalate crystals as they may be sources of calcium (Cromack et al 1977). The production of acids by EM fungi have been shown to have antagonistic effects when grown with soil microbes via the reduction of pH (Rasanayagam & Jefferies 1992), which may indirectly reduce decomposition rates of EM fungal litters by reducing the habitat suitability of the surrounding soil for decomposer microbes.

3. Mycelium morphology

There is large degree of variation in the anatomy of hyphae across species of EM fungi, which can vary in cell wall thickness, branching, and hyphal width (Rillig & Mummey 2006). This variation is compounded by the numerous mycelium morphologies produced by fungi. Most fungi will produce a diffuse mycelium when grown in culture where resources are homogeneously distributed in the medium. Thus, the research examining the decomposition dynamics of fungal tissues has been biased towards lab-grown diffuse mycelium (see Koide & Malcolm 2009; Koide *et al.* 2011; Wilkinson *et al.* 2011; Drigo *et al.* 2012; Fernandez & Koide 2012). EM fungi allocate a great deal of resources to produce specialized structures that carry out certain functions in the life cycle of the fungus such as host resource exchange and interaction (ectomycorrhizas), soil resource acquisition (cords, rhizomorphs, mats), reproduction and dispersal (sporocarps), and dormancy (sclerotia). The physiochemical properties of hyphae are often dictated by the structures in that they compose which in turn likely has implications on the decomposition dynamics of the resulting litter.

3.1 The ectomycorrhiza

The ectomycorrhiza is a unique, composite structure which comprising both fungal and plant tissues. Because of the intimate association between fungus and root significantly alters the chemistry of the root, its decomposition dynamics are also influenced (Langley *et al.* 2006). Fine root production represents a large annual C input into forest ecosystems accounting for approximately one-third of global annual NPP (Jackson *et al.* 1997). Langley & Hungate (2003) highlighted the potential influence of ectomycorrhizal fungal colonization on the decomposition

of fine roots, hypothesizing that ectomycorrhizal colonization would reduce the decomposition rate of fine roots. This hypothesis was later supported by Langley *et al.* (2006) in an experiment examining the decomposition of fine roots of pinyon pine (*Pinus edulis*). With the goal of understanding potential species differences, Koide *et al.* (2011) examined the effects of colonization of different EM fungal species on the decomposition of red pine (*Pinus resinosa*) fine roots. Of the ectomycorrhizas examined in this study, only ectromycorrhizas from a *Suillus* sp. had significantly different decomposition rates from non-mycorrhizal fine roots, which were actually faster than that of non-mycorrhizal fine roots. In a minirhizotron study, Fernandez *et al.* (2013) observed that ectomycorrhizas of *C. geophilum* persisted in the soil 4-10 times longer than other ectomycorrhizas of other species likely due to reduced decomposition rates. Together, these studies have highlighted the importance of fungal species identity to the effect of colonization on fine root decomposition dynamics. Ectomycorrhiza fungi vary widely in their morphology and physiochemical properties. This may explain some of the functional diversity found among EM fungi (Agerer 2001). We argue that these differences in morphology of the ectomycorrhizas might explain some of the variation we see decomposition dynamics. For instance, long distance exploration strategy ectomycorrhizal fungi are often hydrophobic (Agerer 2001) and thus likely to be more resistant to decay than shorter exploration strategy ectomycorrhizal fungi that are hydrophilic.

3.2 Rhizomorphs, cords, and mats

Some Basidiomycete EM fungi produce rhizomorphs and cord structures thought to be important for efficient resource foraging across relatively long distances in the soil. Rhizomorphs

are tube-like structures of linearly aligned hyphae differentiated into the medulla, composed of large diameter hyphae, upwards of 25 µm, encased by smaller diameter, hydrophobic hyphae comprising the cortex (Cairney 1989). These structures are able to conduct water and nutrients across relatively lengthy distances in the soil (Brownlee *et al.* 1983), which can be of particular advantage when water is distributed heterogeneously in soil. There have been accounts that show that these structures persist in the soil for long periods of time, indicating that these are particularly resistant to decomposition (; Treseder *et al.* 2005; Pritchard *et al.* 2008; McCormack *et al.* 2010). The resistance may be due to the hydrophobic surfaces, as well as the relatively low surface area to volume ratio. McCormack *et al.* 2010 found that larger diameter rhizomorphs had shorter persistence times than those with smaller diameters. The effective surface area of the larger diameter rhizomorphs could actually be greater because of a larger lumen, which exposes more tissue to decomposer microbes. Longer persistence could also be the result of these structures living longer or going into a dormant state under unfavorable conditions. Future studies should explicitly examine decomposition rates of these tissues in order to better understand which factors lead to the persistence of these structures in soils.

Ectomycorrhizal fungi can also form dense hyphal mats in the organic layer of forest soils, which can account for ca. 50% of the weight of the layer. These fungal mats are common globally but are particularly prevalent in boreal forest systems dominated by Douglas fir species (*Psuedotsuga* sp.) (Griffiths *et al.* 1990; Griffiths *et al.* 1996). Fungal mats can be rhizomorphic and extremely hydrophobic in nature depending on the species that comprise the mat (Unestam & Sun 1995). The community composition of mat soils was once thought to be dominated by single species of EM fungi but there is now evidence provided by molecular analyses that these communities are more diverse than once thought (Dunham *et al.* 2007). Because of the large

amount of aggregated biomass ectomycorrhizal mats significantly alter the biogeochemistry of the profile they inhabit (Kluber *et al.* 2010).

3.3 Sporocarps and sclerotia

Perhaps the most underappreciated EM fungal litter results from sporocarp production. A great deal of C and N is allocated to the production of these structures in some EM fungal species (Vogt & Edmonds 1980). Vogt *et al.* (1981) found that epigeous sporocarp production was 27-34 kg ha^{-1} and 1-380 kg ha^{-1} (dry weight) in spruce stands in Western Washington, USA . The functional specialization of sporocarps is reflected in their biochemistry. Protein and chitin concentrations of these structures are significantly different from the associated belowground mycelium (Hobbie & Agerer 2010). Thus, these structures may have different decomposition dynamics from the associated belowground mycelium.

The standing biomass of sclerotia produced by the EM fungus, *Cenococcum geophilum*, can be quite dramatic. In a Douglas fir dominated boreal forest Vogt et al. (1981) found quantities that ranged from 2300-3000 kg ha^{-1}. Considering the high production rates of *C. geophilum* sclerotia and the extremely slow decomposition of its ectomycorrhizas due to its heavy melanization, the resulting litter from its sclerotia may represent an incredibly stable C input resulting in significant sequestration of C and nutrients in soils considering their persistence in the soil for extremely long periods of time (Watanabe *et al.* 2007).

4. Diversity of ectomycorrhizal fungal litters

Ectomycorrhizal fungal communities are often highly diverse with species richness exceeding a hundred species in some ecosystems (Dickie 2007). Given the variation in decomposability of EM fungal tissues, understanding the effects of EM fungal diversity on C and nutrient cycling is of great importance. The importance of species differences in decomposition dynamics should not be underestimated for EM fungal litters. The fungi span two Phlya. Differences in their chemical and physical properties can be quite large. It would be unreasonable to expect leaf litter from a pine to decompose at the same rate as that from a maple. It is equally unreasonable to expect EM fungi from different phyla to produce litters that have the identical decomposition dynamics. Thus, the community composition of EM fungal species may have a profound influence on the contribution of EM fungal litters to SOM. Because a single tree may have dozens of EM fungal species colonizing its fine roots, theoretically its fine roots (ectomycorrhizas) can have vastly different decomposition rates as a result of hosting multiple EM fungal species. Additionally, it is not hard to imagine that in a community with low species evenness, a single species could exert considerable control on C and nutrient cycling. For instance, *C. geophilum* is often very abundant in communities (i.e. up to 97 % relative abundance; Trocha *et al.* 2012) and so may result in more SOM accumulation than some other species (Fernandez *et al.* 2013).

Throckmorton *et al.* (2012) examined differences between fungal and bacterial contributions to SOM by measuring the retention of ^{13}C-labeled necromass in soil. The authors found no differences in mean residence times of C in soil between fungal and bacterial groups. Also, fungi from the two sites (California and Puerto Rico) did not differ in their decomposition leading the authors to conclude that site differences were more important than species identity of the fungal necromass in terms of retention of C in SOM. However, the range of species used

was limited to 4 from each site and both amendments were dominated (78% and 87%) by a single genus (*Penicillium*). Given the wide variation in decomposability of fungal tissues, additional studies with greater levels of diversity would be helpful in understanding the contribution to SOM by fungal litters.

In addition to the effects of fungal community structure on C and N cycling, there are other potential interactive effects that litter from different fungal species may have on ecosystem processes. Litter mixtures can exhibit non-additive effects on decomposition; litter mixtures may decompose at greater or reduced rates than expected (Wardle *et al.* 2003; Gartner & Cardon 2004; Chapman & Koch 2007; Jonsson & Wardle 2008). This interactive effect can arise from chemical (Talbot & Treseder 2012), physical (Makkonen *et al.* 2012) and decomposer community (Chapman *et al.* 2013) synergies. Given the close proximity of EM fungal hyphae to other litter types, there are likely to be interactions between the fungi and the other litters during decomposition. Poorer quality litter is expected to decompose at a higher rate in the presence of high quality litter because of priming effects caused by reducing resource limitations, which may increase the production of extracellular enzymes for degrading the relatively recalcitrant compounds. In a microcosm study Wilkinson *et al.* (2011) examined the effects of EM fungal litter mixtures on decomposition rates and found non-additive effects of increasing litter diversity on CO_2 efflux. Overall, the authors found higher CO_2 efflux values when the microcosms were amended with higher levels of diversity (while holding the mass constant). Interactive effects were found among some of the treatments. For instance, the necromass of *Amanita muscaria* and *Paxillus involutus* had higher CO2 efflux values when mixed together than when alone. In contrast, *Cenococcum geophilum* and *Hebeloma crustiforme* litters produced higher CO_2 effluxes when not mixed than when mixed.

5. Extrinsic factors

5.1 Climate

Climatic factors such as temperature and precipitation strongly influence the decomposition rates of litters (Aerts 1997). The effects of temperature on decomposition of litter and SOM are of great interest in the light of climate change. Because the decomposition process is more sensitive to temperature shifts than primary productivity there is concern of positive feedbacks to increases in atmospheric temperature (Davidson & Janssens 2006). Generally speaking, decomposition rates of litter increases with temperature. However, enzyme reactions involved in the breakdown of complex recalcitrant C substrates appear to be more sensitive to temperature than those involved in breakdown of labile substrates (O'Connell 1990; Hobbie 1996; Fierer *et al.* 2005). This is sensitivity is due to the enzyme kinetics; more complex substrates require higher activation energies than less complex substrates (Fierer *et al.* 2005). It stands to reason that sensitivity to temperature among EM litters would have similar dynamics to those of plants.

5.2 Decomposer communities and extracellular enzyme production

Once viewed as homogenous and functionally redundant, soil microbial community composition is now recognized as a major factor determining litter decomposition rates in soils (Strickland *et al.* 2009a,b). Our understanding of the decomposer communities that are responsible for the degradation of fungal tissues are poorly understood when compared to the abundant literature dedicated to those of plant tissues. Early work by Gray & Baxby (1968) found that most microbes were able to utilize chitin as a substrate and a few appears to be chitin specialists. Presumably, soils high in fungal production have decomposer communities that

include a high occurrence of microbes specializing in degrading chitin. For example, in fungal mat communities, chitin and N-acetyl-glucosamine (NAG) is cycled quickly compared to non-mat soils (Zeglin *et al.* 2013), and this may be due to decomposer communities specializing on EM fungal litters in mat soils (Kluber *et al.* 2011).

Because decomposer communities are also at the mercy of the environment, abiotic conditions likely shape the community structure and indirectly effect the decomposition of EM litters. For instance, shifts from the decomposition of lignin to more labile C substrates have been associated with elevated N deposition (Carreiro *et al.* 2000). If the decomposition of melanized EM litter is carried out by similar, if not the same, decomposers (Butler & Day 1998b), there may be similar shifts away from melanin degradation under elevated N.

Important questions regarding the influence of decomposer communities remain. How does the EM fungal community structure influence the microbial decomposer community structure? Does this indirectly influence SOM formation and N mineralization?

5.3 Physical and spatial protection

Relatively labile compounds can persist in SOM for great periods of time as a consequence of physical protection either through soil aggregation or sorption (Nelson et al 1979; Six *et al.* 2006; Grandy & Robertson 2007; Grandy & Neff 2008). Thus, variation in morphology of the extramatrical mycelium could lead to differences in physical protection from the soil. Bogeat-Triboulot *et al.* (2004) found that colonization of *Hebeloma cylindrosporum* had higher soil adhesion to the ectomycorrhiza compared to those formed by *Lactarius bicolor* and *Telephora*

terrestris. It stands to reason that cords and rhizomorphs are less likely to be protected in this manner because of their relatively large diameter.

EM fungi inhabit distinct vertical niches in soil, a likely consequence of resource niche partitioning among EM fungi (Dickie *et al.* 2002; Rosling *et al.* 2003). It is thought that EM fungi positioned higher in the profile rely more on more recent above ground litters containing more labile compounds, while those deeper in the soil profile specialize more on breaking down more recalcitrant compounds (Lindahl *et al.* 2007; Talbot *et al.* 2013). Position in the soil profile also dictates temperature, moisture, decomposer community structure, and various physical soil properties (Schmidt *et al.* 2011). Unlike aboveground litters, EM litters are born and die at specific positions in the soil profile. Thus, there is potential for a species litter decomposition to be controlled largely by where it is located in the soil. Generally, we see slower decomposition and turnover rates of root litter at deeper depths in the soil profile (Gill & Burke 2002; Joslin *et al.* 2006).

Because we see clear vertical niche patterns for many EM fungal species, there may be interesting interactions between litter quality and factors related to soil profile. For instance, an EM fungal species can produce litters of high quality but the decomposition rate of those litters can be counteracted by occurring lower in the soil profile. Moreover, whether sporocarps are epigeous or hypogeous may influence the rate at which they are decomposed. For some species that produce a relatively large number of sporocarps (Kikuchi & Futai 2003), we may see a considerable amount of carbon and nutrients move aboveground from the mycelium belowground where it would presumably decompose at a faster rate.

5.4 Scale

Scale is important to consider when attempting to understand the factors influencing the decomposition dynamics of litters (Aerts 1997). For instance biochemical predictors are often not particularly useful in predicting decomposition dynamics of leaf litter at a global scale (Meentemeyer 1978; Aerts 1997). Most of the work examining EM litter decomposition dynamics has been conducted recently and in relatively few systems. Thus, the influence of scale remains unclear, though it is not hard to imagine similar patterns of influence that are seen in aboveground litters.

6. Synthesis and conclusions

A major finding in the past 10 years is the large contribution of fungal litter to biogeochemical cycles. The primary theme of this work is the importance of species differences in the decomposability of their tissues. This dissertation research suggests that differences in cell wall chemistry plays a large role.

The physiochemical properties of fungal tissue grown in cultures may differ from those grown under more natural conditions. This is why examining the turnover of fungal tissues *in situ* is a necessary goal to gain a better understanding of the differences in persistence of EM litters (Fernandez *et al.* 2013). With next generation minirhizotron imaging technologies that allow finer resolutions (Allen & Kitajima 2013), investigators should be able to identify traits associated with fungi and relate them to persistence in the soil. This will also allow for finer contrasts between EM fungal structures. Finally, these tools should be useful in identifying extrinsic factors controlling decomposition rates of EM fungal litters. A major goal of this area

of research will be to explore the linkages between EM fungal community structure and biogeochemical cycling. For instance, do mono-dominant communities have drastically different contributions to SOM formation than more diverse communities? Ultimately, a major goal will be to link factors controlling the decomposition of these litters to SOM formation.

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Figures and Tables

Figure 5.1. A schematic diagram depicting the production (green), turnover (blue), and decomposition (red) of ectomycorrhizal (EM) fungal litters.

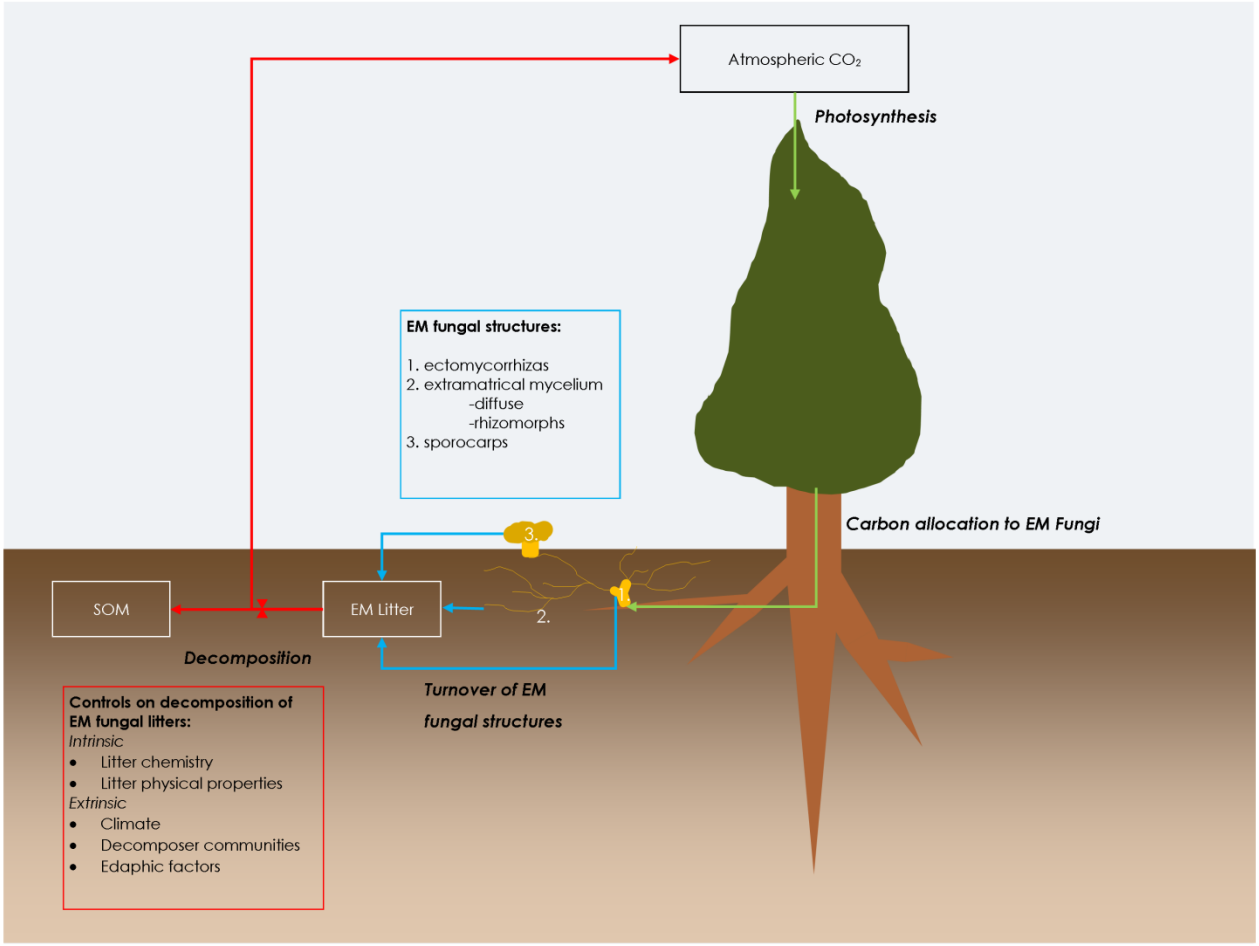

Table 5.1. Major biochemical components of ectomycorrhizal fungal cells, the nutrients contained in those components, their function, and their relative lability during decomposition. Uncertainties are denoted with a (?).

Substrate	Nutrients	Component function	Enzymatic attack	Lability
Cytoplasm				
Solubles	C,N,P	Various	N/A	High
Glycogen	C	Energy storage	Hydrolytic	High
Lipids	C	Energy storage & Structural	Hydrolytic	High-Medium
Protein	C,N,P	Various	Hydrolytic	Medium
Cell Wall				
Glucans				
α- glucans	C	Matrix	Hydrolytic	High (?)
β- glucans	C	Structural	Hydrolytic	Medium (?)
Chitin	C & N	Structural	Hydrolytic	Medium
Protein				
Glycoproteins	C & N	Matrix, signaling	Hydrolytic	Medium (?)
Hydrophobins	C & N	Water proofing	Hydrolytic(?)	Low (?)
Melanin	C	Structural & Protectant	Oxidative	Low

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CURRICULUM VITAE

EDUCATION

The Pennsylvania State University
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PUBLICATIONS

Fernandez, C.W. & Koide, R.T. (*In review*). Is melanin a major controller of the decomposition of ectomycorrhizal fungal litter? *Ecology*

Koide, R.T., **Fernandez, C.W.**, & Malcolm G.M. (2013). Determining place and process: functional traits of ectomycorrhizal fungi that impact both community structure and ecosystem function. *New Phytologis*t. DOI: 10.1111/nph.12538

Fernandez, C.W. & Koide, R.T. (2013) The function of melanin in the ectomycorrhizal fungus *Cenococcum geophilum* under water stress. *Fungal Ecology*. 6:479-486 DOI:10.1016/j.funeco.2013.08.004

Fernandez, C.W., McCormack, M.L., Hill, J.M, Pritchard, S.G. & Koide, R.T. (2013). On the turnover of *Cenococcum geophilum* ectomycorrhizas and its implications for forest carbon and nutrient cycles. *Soil Biology & Biochemistry* 65:141-143.

Fernandez, C.W. & Koide, R.T. (2012). The role of chitin in the decomposition of ectomycorrhizal fungal litter. *Ecology*. 93: 24-28. DOI: 10.1890/11-1346.1

McCormack, M.L. & **Fernandez, C.W.** (2011). Measuring and modeling roots, the rhizosphere, and microbial processes belowground. *New Phytologist*. 192: 573-575

Koide, R.T., **Fernandez, C.W.,** & Peoples M. (2011). Can ectomycorrhizal colonization of *Pinus resinosa* roots affect their decomposition? *New Phytologist*. 191: 508-514. DOI: 10.1111/j.1469-8137.2011.03694.x

Koide, R.T., **Fernandez, C.W.,** & Petrakob K. (2011). General principles in the community ecology of ectomycorrhizal fungi. *Annals of Forest Science*. 68: 45-55. DOI: 10.1007/s13595-010-0006-6

PRESENTATIONS

Fernandez, C.W. & R.T. Koide (2013). Towards a mechanistic understanding of ectomycorrhizal litter decomposition dynamics. Soil Ecology Society Conference, Camden, NJ.

Fernandez, C.W. & R.T. Koide (2012). Melanin: a functional trait conferring tolerance to water stress in ectomycorrhizal fungi. The Ecological Society of America Annual Meeting, Portland, OR

Fernandez, C.W. & R.T. Koide (2011). The role of chitin in the decomposition of ectomycorrhizal fungal litter. The Ecological Society of America Annual Meeting, Austin, TX.

Fernandez, C.W. (2011) Does chitin reduce the decomposability of ectomycorrhizal necromass? Program Seminar, Ecology Program, The Pennsylvania State University.

Fernandez, C.W. (2010) Mycorrhizal fungal and their ecology**.** Guest lecture (Concepts in Ecology), The Pennsylvania State University.

Fernandez, C.W. (2010) Functional traits of ectomycorrhizal fungi and water stress. Program Seminar, Ecology Program, The Pennsylvania State University.

AWARDS & GRANTS

Soil Ecology Society Conference Student Oral Presentation Award (Soil Ecology Society) Second Place 2013
Frank A. Andersen Ecology Travel Award (Pennsylvania State University) 2012
College of Agricultural Sciences Graduate Student Competitive Grant (Pennsylvania State University) 2011
Alfred P. Sloan Fellowship (Alfred P. Sloan Foundation) 2009-2013
The Huck Institutes of the Life Sciences Fellowship (Penn State University) 2008

PROGRAM, DEPARTMENT, COLLEGE & UNIVERSITY SERVICE

President, Ecology Graduate Student Organization (Pennsylvania State University) 2012- 2013
Curriculum Committee Representative, Ecology Graduate Program (Pennsylvania State University) 2009-2010
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Organizer, Concepts in Ecology Course (Pennsylvania State University) Fall 2009