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Distributions of fungal melanin across species and soils

Cheta E. Siletti, Carolyn A. Zeiner, Jennifer M. Bhatnagar*

Dept. of Biology, Boston University, Boston, MA 02215, United States

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ABSTRACT

Soil is one of Earth's largest carbon (C) sinks, and the diverse community of fungi it houses may affect soil C storage through the biosynthesis of recalcitrant cell wall polymers like melanin. We tested the hypotheses that (1) specific biological features of fungi - evolutionary history, functional guild, growth rate, and functional gene abundance in fungal genomes - predict fungal melanin content across species and (2) the abundance of melanin-producing fungi in soil is related to soil C storage and oxidative enzyme activity by colorimetrically assaying melanin in hyphal tissue of 62 fungal species. We found no phylogenetic signal for melanin content across the species used in our study. Instead, hyphal melanin content varied across fungal species, correlating with 177 protein domains encoded in fungal genomes. Melanin concentrations were positively correlated with protein domains involved in biosynthesis of phenolic melanin precursors, as well as primary and secondary metabolite biosynthesis, cell signaling, and oxidation-reduction reactions. By contrast, hyphal melanin content was negatively correlated with protein domains involved in DNA replication processes and stress response, as well as hyphal growth rate, suggesting a physiological tradeoff between melanin biosynthesis and cellular growth. Estimated melanin content of soil was positively correlated with total soil C and soil peroxidase activity, suggesting that fungal melanin may influence soil C cycling processes.

1. Introduction

Microbes impact carbon (C) in soil by acting as decomposers and plant root associates, as well as by sequestering fixed C in their biomass. In high-latitude ecosystems, much of soil C could be fungal in nature (Clemmensen et al., 2013). However, the biochemical mechanisms by which fungi influence C sequestration in soil are not well understood. Up to several hundred species of fungi, differing in chemical composition and biochemical activity, may exist in a single gram of soil (Taylor et al., 2010). While many studies have mapped decomposition activities onto the taxonomic diversity of fungal communities (Hanson et al., 2008; McGuire et al., 2010; Setala and McLean, 2004; Talbot et al., 2015), less attention has been paid to how the diversity of fungal species affects soil C sequestration, what roles individual species play, and which biological aspects of these species predict soil C stores.

One way in which fungal diversity may affect soil C is through melanin pigment production (Treseder and Lennon, 2015). Melanin is a large class of pigment molecules that is stored in the fungal cell wall and has a wide variety of functions; it can confer resilience to pathogens, protection from UV light, and tolerance to desiccation and osmotic stress (Bell and Wheeler, 1986; Khajo et al., 2011). Because of its recalcitrance, fungal melanin is expected to have a positive effect on soil C storage (Bell and Wheeler, 1986). Indeed, decomposition of fungal tissue can decline as melanin content increases (Fernandez and Koide, 2014). Melanin content of fungal hyphae can differ among species (Bell and Wheeler, 1986), yet it is not yet clear how melanin production, or the effect of this species trait on soil C, varies across a diversity of fungal taxa.

Closely related fungal taxa may have similar levels of melanin production in their tissues, which could allow for prediction of community-level C storage based on genetic information alone. Across the fungal kingdom, presence of a cell wall is restricted to younger lineages (Treseder et al., 2014), such that melanin concentrations may vary across fungal phyla. Ascomycetous fungi primarily produce melanin from dihydroxynaphthalene (DHN) monomers, synthesized from secondary metabolites derived from acetate (Butler et al., 2009), while basidiomycetous fungi primarily produce DOPA melanin, synthesized from tyrosine (Figs. S1a and S1b) (Singh et al., 2013). These observations suggest conservation of cell wall traits throughout the evolutionary history of fungal species (Swenson et al., 2007; Zanne et al., 2005).

* Corresponding author.
E-mail address: jmtalbot@bu.edu (J.M. Bhatnagar).

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However, fungi with different trophic strategies (i.e. pathotrophs, saprotrophs, or symbiotrophs) or functional guilds (i.e. white rot, brown rot, or ectomycorrhizal) may also vary in their tissue melanin content. For instance, the fungal rice blast pathogen *Magnaporthe grisea* utilizes melanin to assist in penetrating plant tissue (Howard and Valent, 1996), while soft rot fungi secrete melanins when attacking wood (Nilsson, 2009). These different "functional guilds" of fungi span the fungal phylogeny (Hibbett and Donoghue, 1998, 2001; Tedersoo et al., 2010), such that phylogenetic relatedness of species may be of secondary importance to functional guild or trophic strategy in determining melanin content of a species. Indeed, functional guild classification can be a better predictor of decomposition-related traits in fungi than phylogenetic relatedness (Talbot et al., 2015; Treseder and Lennon, 2015).

Melanin production by fungi may also vary in a systematic way across species if it results in a tradeoff with other physiological characteristics of fungi that affect fitness. Melanin is a chemically complex molecule (Nosanchuk et al., 2015) consisting of phenolic moieties (such as quinone and its derivatives) surrounded by protein, carbohydrate, and lipid molecules (Bell and Wheeler, 1986), all of which are energetically expensive to produce. Production of such complex secondary metabolites is often observed to come at a cost to growth (Schlatter and Kinkel, 2015). For example, increased melanin pigmentation in a butterfly model results in slower growth and smaller size (Blois, 1978; Windig, 1998), presumably because of a tradeoff between growth and melanization. In addition, melanin can prevent growth of some fungal species by scavenging heavy metals (Gadd, 1993) and reducing secretion of extracellular enzymes involved in resource acquisition (Henson et al., 1999). Fungal growth can correlate with soil C stabilization through mechanisms such as aggregate formation (Lehmann and Rillig, 2015; Rillig et al., 2015; Six et al., 2004). If melanin is inversely correlated with fungal growth, however, the chemical composition – or "quality" – of fungal tissue may play a more prominent role in soil C storage than total C inputs to soil (Clemmensen et al., 2013; Cotrufo et al., 2015).

Variance in fungal melanin production, as well as a potential growth tradeoff, may be predicted by biochemical functions encoded in fungal genomes. For example, for fungi in the Ascomycota, melaninization is decreased when the genes for tetrahydroxynaphthalene reductase and polyketide synthase 1 (PKS1) are downregulated (Mishra and Singh, 2015; Yu et al., 2015). Furthermore, mutations in the gene encoding scytalone dehydratase, an enzyme in the DHN melanogenesis pathway, decreases resistance of fungi in the Ascomycota to fungicides (Yamada et al., 2004), indicating that proper functioning of this gene is essential to protection of fungal cells by melanization. Functional gene abundance (as gene counts per protein) can be predictive of extracellular enzyme activity produced by decomposer fungi (Baldrian et al., 2011; Schneider et al., 2012) and can effectively delineate specific functional guilds of fungi (Floudas et al., 2012; Kohler et al., 2015; Riley et al., 2014; Talbot et al., 2015). Functional genes could also be a stronger predictor of melanin concentrations in fungi than functional guild alone, as functional genes can incorporate not only the biochemical machinery responsible for an organism's phenotype, but also aspects of evolutionary history of the organism.

The objective of this research was to determine which biological aspects of fungi predict melanin production across fungal taxa and if melanin content of fungal species could predict community-level C cycling processes in soil. We hypothesized that 1) melanin production in hyphae varies across the fungal phylogeny, with more closely related species producing similar amounts of melanin, 2) functional guild/trophic strategy of fungi predicts hyphal melanin content, with pathotrophs having the highest hyphal melanin content, 3) a tradeoff exists between growth rate and melanin production across species, such that gene abundance for enzymes involved in DOPA or DHN melanogenesis could predict melanin content, and 4) melanin content of fungal species within soil communities could predict soil enzyme activities and C content. To test these hypotheses, we measured melanin content of hyphal tissue of 62 fungal species. We also examined the relationships between melanization and growth rate, as well as between melanization and genes coding for enzymes involved in DOPA and DHN melanogenesis for 29 species that had publically-available sequenced genomes. Finally, we quantified the effects of species' melanin content on soil C stocks and extracellular enzyme activities in soil using previously published data on total soil C, extracellular enzyme activity, and relative abundance of fungal species in field soil (Talbot et al., 2014).

2. Materials and methods

2.1. Tissue preparation and melanin assay

Hyphal tissue for 62 fungal species (Table S1) was grown on liquid modified Melin-Norkrans (MMN) media (Marx, 1969) for 100 days. Species spanned 52 genera, 35 families, 15 orders, and three phyla of fungi, although most species were in the Agaricomycetes class (50/62) of the Basidiomycota (55/62). Tissue was then filtered by vacuum filtration onto a nylon filter (0.45 μM), rinsed twice with deionized water, and air-dried. Tissue was visually inspected for contamination, and to prepare samples for melanin assay, tissue was scraped off of filters and weighed into individual test tubes.

Two replicate cultures of each species were assayed for melanin concentrations using the Azure A dye procedure following Fernandez and Koide (2014). Tissue was incubated in 3 mL Azure A stock dye for 90 min in the dark, the solution was filtered, and absorbance was measured at 610 nm. Melanin amount was calculated using a standard curve prepared with pure melanin extracted from *Cenococcum geophilum* hyphal tissue, following Fernandez and Koide (2014). This assay is ideal for measuring fungal melanin because of the strong affinity of the dye for melanin particles and the short, 90-min run time (Butler and Lachance, 1987).

2.2. Phylogenetic analysis

We downloaded 28S rRNA sequences for 58 of the 62 species used in the study (Table S1) from the SILVA ribosomal RNA database v. 123 (Quast et al., 2013). Sequences were aligned to the SILVA SEED LSU nucleotide alignment and a maximum-likelihood tree was created using the fast maximum-likelihood stepwise-addition algorithm in GARLI (Zwickl, 2006) with 36 positive constraints (Fig. 1). Eight eukaryotic outgroup species were included from a previously published multi-gene tree for Kingdom fungi (James et al., 2006). Branch lengths were calculated by the Grafen (1989) method with the compute.brlen command in the ape package in R (Paradis, 2012). To test for a phylogenetic signal to melanin production, Blomberg's K statistic (Blomberg et al., 2003) was calculated for hyphal melanin concentration using the multiPhylosignal command in the Picante package in R (Kembel et al., 2010).

2.3. Functional guild analysis

To test for functional guild effects on hyphal melanin concentrations, we conducted one-way analysis of variance with functional guild classification as the independent variable and melanin content as the dependent variable. We tested both broad trophic strategy (pathotrophs, saprotrophs or symbiotrophs) and specific functional guild (white rot, brown rot, ectomycorrhizal, other saprotroph, fungal/plant pathogen) effects on hyphal melanin concentrations. Fungal taxa were assigned to trophic strategies and