Root diameter predicts the extramatrical hyphal exploration distance of the ectomycorrhizal fungal community

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Abstract. Locally coexisting tree species of temperate forests often vary widely in the diameter of their absorptive roots, resulting in contrasting strategies of root foraging within soil nutrient hot spots. We hypothesized that root diameter would also influence the extramatrical hyphal exploration distance of the mycorrhizal fungal community due to coevolution of the plant and fungal partners leading to functional complementarity. We collected absorptive roots from mature trees of nine ectomycorrhizal tree species in replicated monoculture plots of a plantation where the soil is relatively homogeneous. The identities of ectomycorrhizal fungal taxa and their relative abundances were determined by DNA sequencing. The hyphal exploration type (i.e., contact, short-distance, medium-distance or long-distance) was assigned to each taxon, allowing us to calculate an index of the abundance-weighted mean extramatrical hyphal exploration distance of the fungal community. Overall, there was a significant, positive correlation between root diameter and our index of hyphal exploration distance such that tree species with the thicker roots were associated with fungi with the longer hyphal exploration distance. Moreover, we found that root diameter negatively correlated with the proportion of contact ectomycorrhizal fungal taxa and positively correlated with the proportion of medium-distance ectomycorrhizal fungal taxa. Our results suggest that among temperate ectomycorrhizal tree species, root diameter selects for communities of mycorrhizal fungi of specific exploration distance in a way that is consistent with root-fungal functional complementarity in nutrient foraging.

Key words: ectomycorrhizas; hyphal exploration type; Illumina sequencing; root diameter; root functional traits; root length density; tree species diversity.

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INTRODUCTION

Plant species have evolved a wide range of root functional traits and strategies in order to absorb nutrients (Grime 2001, Chen et al. 2013, Bardgett et al. 2014, Ma et al. 2018). Among these, the diameter of absorptive roots strongly correlates with other root traits that influence nutrient absorption, such as branching intensity. Generally, thin roots branch more in response to nutrient hot spots in the soil than thick roots (Comas and Eissenstat 2009, Eissenstat et al. 2015). Therefore, root diameter can be used to predict a plant’s capacity to exploit spatial nutrient heterogeneity (Eissenstat et al. 2015, Liu et al. 2015, Chen et al. 2016, 2017, 2018, Cheng et al. 2016). Root diameter also largely determines root construction efficiency and contributes to the
overall root economics strategy of a plant species as indicated by root turnover rate (Eissenstat 1992, McCormack et al. 2012, Mommer and Weemstra 2012).

Variation among plant species in root function may be compensated for by significant variation in function among species of mycorrhizal fungi (Koide 2000). Studies have revealed large variation among ectomycorrhizal (EM) fungal taxa in many functional traits (Coleman et al. 1989, Fernandez and Koide 2014, Koide et al. 2014, Clemensen et al. 2015, Trocha et al. 2016), including hyphal exploration type (Agerer 2001, Tedersoo and Smith 2013), which is related to the distance hyphae extend from the root and thus the spatial scale at which they are able to forage. Therefore, mycorrhizal hyphal exploration type may determine the degree to which the fungi can substitute for roots when foraging for nutrients in spatially heterogeneous soils. By exploring large volumes of soil, EM fungal species with a long-distance exploration strategy may be able to compensate for the relative inability of slow-growing, large-diameter roots to exploit nutrient hot spots (Chen et al. 2017). For example, *Suillus*, a genus that frequently produces rhizomorphic hyphae that explore large volumes of soil at great distances from the roots (Agerer 2001, Tedersoo and Smith 2013), is largely restricted to host species in the Pinaceae (Bruns et al. 2002), which often possess comparatively large-diameter absorptive roots (Chen et al. 2016). In contrast, for plant species with relatively small diameter roots (e.g., species in the Fagaceae) that are capable of rapidly exploiting nutrient hot spots by root proliferation (Chen et al. 2017), it may be beneficial to associate with fungi possessing a short-distance exploration strategy, requiring smaller amounts of resources for construction and maintenance (Ekblad et al. 2013). Therefore, there may be selection for plant–fungus specialization that reduces the probability of forming less beneficial combinations (Kiers et al. 2011, Hortal et al. 2017) of root diameter and hyphal exploration type.

In this study, we investigated the relationship between root diameter and EM hyphal exploration type. We hypothesized that tree species with thicker absorptive roots associate with communities of EM fungi providing longer hyphal exploration distances. Root diameter is often negatively correlated with short-term root growth rate (Eissenstat 1991, Eissenstat et al. 2015), leading to an effect on root length density. Because root length density may independently influence hyphal exploration type (Peay et al. 2011), we also examined this relationship to determine whether it confounded the relationship between root diameter and hyphal exploration distance.

**Methods**

**Site description and root collection**

We collected root samples from mature trees in a common garden plantation located in central Pennsylvania, USA (40°42’ N, 77°57’ W). There are 16 different tree species in the plantation, nine of which are EM and seven of which are arbuscular mycorrhizal. Six trees of a given species are planted within each of eight replicate plots, arranged in a randomized, complete block design. Each plot was isolated from neighboring plots by a distance of 5 m, and a plastic sheet barrier was installed vertically in the soil between plots to a depth of 1 m in order to minimize root growth from plot to plot. Details of plot design were previously described (McCormack et al. 2012, Chen et al. 2016). Because the reference database of arbuscular mycorrhizal fungal hyphal exploration distance is extremely limited, we selected only EM tree species for this study (Table 1). The relatively homogeneous soil in the plantation provided an ideal opportunity

| Species Abbreviations | Diameter (mm) | RLD (cm/cm³) |
|-----------------------|--------------|-------------|
| Carya glabra          | Cagl         | 0.19 ± 0.02 | 0.81 ± 0.08 |
| Quercus alba          | Qual         | 0.19 ± 0.01 | 0.80 ± 0.10 |
| Quercus rubra         | Quru         | 0.21 ± 0.01 | 1.27 ± 0.11 |
| Betula alleghaniensis | Beal         | 0.24 ± 0.01 | 0.40 ± 0.09 |
| Populus tremuloides   | Potr         | 0.24 ± 0.01 | 1.54 ± 0.16 |
| Betula lenta          | Bele         | 0.29 ± 0.01 | 0.19 ± 0.05 |
| Picea rubens          | Piru         | 0.45 ± 0.03 | 0.68 ± 0.13 |
| Pinus virginiana      | Pivi         | 0.46 ± 0.02 | 0.24 ± 0.03 |
| Pinus strobus         | Pist         | 0.64 ± 0.05 | 0.85 ± 0.07 |

*Note: Abbreviations for each species are also given.*
to study the effects of host plant functional traits in the absence of large environmental variation.

In late July 2014, four intact root branches (15–20 cm long) were sampled from the 0–10 cm soil layer, each from a different random location within each plot. Because seasonality may influence some of the root characters and the associated mycorrhizal fungal community (Voříšková et al. 2014, Zadworny et al. 2015), we collected all samples at the same time. A subset of approximately ten short root branches (consisting of the first three root orders, sensu McCormack et al. 2015) from each plot was selected for scanning on a desktop scanner, and images were processed with WinRHIZO (Regent Instruments) to determine average root diameter (Table 1). In order to verify the potential bias in diameter determined by scanning and WinRHIZO, we randomly select 3–5 root branches of each tree species and measured the diameter of first-, second-, and third-order roots under a microscope. Root length densities (0–10 cm depth) were also determined within the plots of each species in the summer of 2014 by root coring, scanning, and length determination by WinRHIZO (Table 1).

Molecular methods

Twenty root segments, usually <1 cm in length and including the first two root orders, were dissected from each sampled root branch, since the first two orders of roots comprised the majority of the mycorrhizal roots (Guo et al. 2008). All root segments from a given plot were combined prior to DNA extraction, resulting in 80 root segments per sample. DNA was extracted using MoBio Power Soil DNA extraction kits (MoBio Laboratories, Carlsbad, California, USA), following the manufacturer’s recommendations.

Following DNA extraction, the ITS1 region was amplified using primers of ITS1F and ITS2 (CTTGGTCTTTAGAGGAACTTAA/GCGTTCTT CATGATGC, White et al. 1990). Forward primers were linked to an Illumina adapter with the sequence 5′-ACACTCTTTCCCTACACGAC GCTCTTCCGATCT. Reverse primers were linked to an adapter with the sequence 5′-TGACTGGAGTTCAGACGTGTGCTCTTCCGAT C. The PCR products were sent to the DNA Sequencing Facility at the University of Wisconsin-Madison where a library was prepared by adding an index sequence in a second PCR step to each sample. The library was sequenced on the MiSeq platform with 2 × 300 bp pair-end reading.

Pair-end reads were first merged using PAN-DAsq assembler (Masella et al. 2012) and then quality-filtered and processed using QIIME v1.8.0 (Caporaso et al. 2012). Sequences were clustered into operational taxonomic units (OTUs) using an open reference-based (i.e., reference-based + de novo) approach with the UCLUST algorithm (Edgar 2010) and a 97% similarity threshold. The number of reads of an OTU was used to represent its initial relative abundance within the root tips. The UNITE (Abarenkov et al. 2010) database was used as the reference database for taxonomy assignment via the RDP classifier (Wang et al. 2007) using a 0.7 confidence threshold. Because the primer pair ITS1F-ITS2 amplified all fungal DNA, we checked the trophic status (EM, non-EM, unknown) of the assigned genus by referencing online databases such as UNITE (http://unite.ut.ee/), DEEMY (http://www.deemy.de/), and other literature (Tedersoo and Smith 2013, Trocha et al. 2016) to include only EM OTUs in subsequent analyses. We assumed that EM status is conserved within a fungal genus.

By combining into a single sample the 80 root tips from any particular plot, we were unable to determine the effect of diameter of individual root segment on the functional composition of the EM fungal community. Instead, we determined the effect of species-specific root diameter, with the understanding that root diameter may vary widely within plant species. We are also aware that the use of read number may be a biased metric for characterizing EM fungal community structure because DNA of some fungal species may be easier to extract and amplify than others, and because species may differ in the amount of DNA they contain for a given degree of colonization.

The EM fungal OTU tables were rarefied to remove the influence of sequencing depth variation among samples. In each sample, sequencing reads were rarefied to 1000. Hyphal exploration types were assigned to genus based on the DEEMY database and references in Tedersoo and Smith (2013). We assumed that hyphal exploration types are conserved within a genus, although clearly some genera (Russula, Tomentella,
and *Lactarius*) have multiple exploration types (Tedersoo and Smith 2013). In such cases, we assigned exploration types based on Peay et al. (2011) because his site and ours are North American at similar latitude and with a shared host genus (*Pinus*). As reported in Peay et al. (2011), the dominant *Russula* in our study (*R. amoenolens*, Appendix S1: Table S1) has a short-distance hyphal exploration type, while *Lactarius luculentus* and all six species of *Tomentella* possess a medium-distance exploration type. We understand that exploration type may vary among sites with different environments, but it is currently impossible to find reference database of exploration type for all relevant fungal species in one particular site. For some OTUs, there was insufficient information based on genus-level resolution to assign hyphal exploration type and these taxa were left out of subsequent analyses (~0.25% of all OTU abundance). The data of relative abundance of each exploration type across plots of all tree species are accessible in Figshare (https://doi.org/10.6084/m9.figshare.6030776).

**Statistical analyses**

The composition of the EM fungal community based on hyphal exploration type as opposed to taxon was visualized using non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis distances (1000 iterations; Bray and Curtis 1957, Oksanen et al. 2015). We tested for significant differences in the distribution of hyphal exploration types among tree species, using PERMANOVA with Bray-Curtis distances matrices (Anderson 2001, Oksanen et al. 2015). To quantitatively examine the relationship between root diameter and hyphal exploration type, we conducted simple regression on the proportion of each hyphal exploration type with root diameter across tree species. Moreover, because exploration type is not a quantitative concept, we assigned an arbitrary coefficient for each exploration type but following the order of presumed hyphal length (i.e., contact < short-distance < medium-distance < long-distance; Agerer 2001). We assigned the type with the shortest distance exploration type as opposed to contact type EM fungi and significantly and positively correlated with the proportion of medium-distance type EM fungi, but
the proportions of short-distance and long-distance types were not significantly correlated with root diameter (Fig. 2). Overall, root diameter was significantly and positively correlated with the index of community extramatrical hyphal exploration distance (Dis) at the plant species level ($r^2 = 0.58$, $P = 0.017$, Fig. 3). There was also a positive correlation between root diameter and Dis at the plot level, but much less variation in Dis was explained by root diameter, and the relationship was only marginally significant ($r^2 = 0.04$, $P = 0.10$; Appendix S1: Fig. S1). Root length density did not correlate with Dis across tree species ($P = 0.95$, Fig. 4). In addition, Dis did not differ significantly between angiosperm hosts and gymnosperm hosts (Appendix S1: Fig. S2).

**Discussion**

Despite the critical role of mycorrhizal fungal functional traits in a variety of ecosystem functions and processes (Read and Perez-Moreno 2003, Teste et al. 2010, Clemmensen et al. 2013, 2015, Koide et al. 2014, Treseder and Lennon 2015, Fernandez and Kennedy 2016), their linkage to commonly measured root functional traits has rarely been studied. Root traits are far more easily documented than are functional traits of mycorrhizal fungi (Iversen et al. 2017). Therefore, such linkages would simplify our ability to predict certain ecosystem functions and ecosystem responses to, for example, environmental change. We found that root diameter was positively correlated with the hyphal exploration distance of the EM fungal community. This is consistent with our hypothesis that for trees with thicker absorptive roots, there is selection for associating with EM fungi with longer exploration hyphae, allowing such tree species to indirectly exploit nutrient hot spots.

The linkage between root diameter and extramatrical hyphal exploration distance provides a potential pathway to predict mycorrhizal-mediated ecosystem functions because hyphal exploration distance may correlate with other EM fungal traits, such as hydrophobicity and the rate of hyphal turnover (Hobbie and Agerer 2010, Ekblad et al. 2013). The EM fungi that explore and transfer nutrients over relatively large distances often produce rhizomorphs (Agerer 2001) that can persist for several months or even years (Pritchard et al. 2008). However, fungal species with the long-distance hyphal exploration type were relatively rare (~5\% on average), preventing a test of the response of rhizomorph abundance to root diameter. In this study, the larger Dis was primarily due to greater relative frequency of the medium-distance exploration type and lower relative frequency of the contact exploration type (Fig. 2).

The distribution of EM functional traits, such as hyphal exploration type, may be influenced by factors other than root diameter. For example, hyphal exploration distance was negatively correlated with root length density in *Pinus miricata* stands (Peay et al. 2011), although this was not true in *Quercus ilex* stands (Shahin et al. 2013). Therefore, we determined whether the significant root diameter effect on hyphal exploration distance as seen in our study was confounded by a root length density effect on hyphal exploration distance. There was not a significant correlation...
between root length density and hyphal exploration distance (Fig. 4), suggesting that the relationship between hyphal exploration distance and root diameter was not confounded by differences in root length density. In addition, we did not observe a significant difference in hyphal exploration distance between conifers and angiosperms, suggesting that our results are not confounded by phylogeny (Appendix S1: Fig. S2). Nevertheless, because of the relatively modest number of tree species studied, caution is needed in extrapolating our results.

Despite the existence of the significant correlation between root diameter and hyphal exploration distance, there was significant variation in hyphal exploration distance within an individual tree species (Fig. 1; Appendix S1: Fig. S1), suggesting that selection for complementarity in nutrient foraging was not an overriding consideration in this study. This could result from bet-hedging (Lekberg and Koide 2014) in which selection may occur simultaneously for potentially opposite fungal traits. For example, the traits of fungi selected for root pathogen protection (Sikes et al. 2009, Wehner et al. 2010) may be at odds with those selected for nutrient foraging from heterogeneous soils. Also, the consequences of selection may be drastically reduced if the realized fungal community structure is

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**Fig. 2.** Relationship between species-specific root diameter and the relative proportion of each ectomycorrhizal hyphal exploration type. Only significant linear regression was shown.

**Fig. 3.** Relationship between species-specific root diameter and the index of averaged exploration distance at the community level (Dis). Root diameter and Dis were averaged for each tree species. Significant linear regression was shown ($Y = 2.02 + 2.38X$, $r^2 = 0.59$, $P = 0.017$).
constrained by priority effects (Kennedy et al. 2009).

Our experimental system was a plantation in which the soil may be more homogeneous than in most natural forests. While this homogeneity may have been helpful in reducing the required number of replicates (Kranabetter et al. 2009, Shahin et al. 2013, Ostonen et al. 2017, Pena et al. 2017), it may also have reduced the need for and expression of functional complementarity between fungi and plants in terms of nutrient foraging. Nevertheless, our results do suggest that root diameter is one of many possible factors that influence the traits of EM fungal communities.

Root diameter was determined by scanning, and the length-weighted average diameter was calculated in WinRHIZO. Determination of root diameter by scanning may differ from microscopic determination, but scanning is simpler and widely used for a broad range of tree species (Comas and Eissenstat 2009, Gu et al. 2014, Chen et al. 2016, Cheng et al. 2016). Moreover, we have shown that for the nine EM tree species of this study, average diameters of the first three root orders determined by scanning were within the range of root diameters determined by microscopy and were highly correlated with diameters of first-, second-, and third-order roots determined by microscopy (Appendix S1: Fig. S3).

It is possible that the thickness of EM mantles introduces error in the determination of root diameter, and we made no attempt to subtract the mantle thickness to obtain the true root diameter. But EM colonization of fine roots is very frequent in the nine tree species of this study, and colonization frequently results in a number of changes in root anatomy and morphology, notably an increase in diameter (Smith and Read 1997), so it is not clear what true root diameter means. We argue that because the natural condition of fine roots of these species is mycorrhizal, the diameter of the mycorrhiza (the composite organ) is the relevant diameter, and it is the diameter that all other researchers have measured when establishing relationships between diameter and other root system traits.

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**Supporting Information**

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2202/full