Dual-mycorrhizal colonization is determined by plant age and host identity in two species of *Populus*

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Abstract. Plants have evolved symbioses with mycorrhizal and endophytic fungi that are essential for their growth and survival. While most plants associate with a single guild of mycorrhizal fungi, a select group termed "dual-mycorrhizal plants" associate with both arbuscular mycorrhizal and ectomycorrhizal fungi. Although a shift from predominance of arbuscular mycorrhizal to ectomycorrhizal colonization with plant development has been demonstrated on other dual-mycorrhizal hosts, it is not known how mycorrhizal colonization shifts with plant age in *Populus* species. We performed a controlled growth experiment with natural field-sourced inocula to test for age-dependent shifts in fungal colonization rates and for host-specific patterns of colonization in two species of *Populus* (*P. tremuloides* and *P. trichocarpa*). We found that only *P. trichocarpa* displayed dual-mycorrhizal colonization, while *P. tremuloides* associated with ectomycorrhizal fungi, but not arbuscular mycorrhizal fungi. Both guilds of mycorrhizal fungi increased in abundance with plant age, while root endophytic fungal colonization decreased. Many of the early-colonizing endophytic fungi that we documented have strong saprotrophic capabilities, which may be an important trait for fast colonization. Dark septate endophytes were more abundant than either guild of mycorrhizal fungi, and are likely to be functionally important members of the *Populus* root fungal community. Our findings represent a novel pattern in the development of dual-mycorrhizal colonization and illustrate that *Populus* species vary in their association with arbuscular mycorrhizal fungi. Our results also highlight the importance of dark septate endophyte colonization dynamics on dual-mycorrhizal plants.

Keywords. Dual-mycorrhizal, dark septate endophytes, *Populus*, succession, ectomycorrhizae, arbuscular mycorrhizae

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Introduction

Plants have evolved symbioses with diverse mycorrhizal fungal symbionts that perform important functions in the plant root environment, including nutrient transfer and protection from abiotic and biotic stress (Behie & Bidochka, 2014; Branco et al., 2022). The two most common guilds of mycorrhizal fungal symbionts are arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi, which colonize 72% and 2% of vascular plant species respectively (Brundrett & Tedersoo, 2018). Although associations with both groups of fungi have evolved independently across multiple plant lineages, plant mycorrhizal type is generally shared among species within the same genus and sometimes across higher taxonomic levels, indicating some degree of phylogenetic conservation (Brundrett & Tedersoo, 2018; Meng et al., 2023). However, an increasing number of exceptions to this pattern of phylogenetic conservation have been reported, with reports of many plant species nested within clades that predominantly exhibit a different mycorrhizal type (Bueno et al., 2019). While vascular plants associate with a single guild of mycorrhizal fungi, there are plants termed "dual-mycorrhizal" that can associate with both AM and EM fungi. Although this group of dual-mycorrhizal plants is relatively small, estimated at between 1500 to 7300 plant species or <3% of flowering plants (Brundrett & Tedersoo, 2019; Teste et al., 2020), it includes dominant trees on multiple continents, such as *Populus* spp. and *Eucalyptus* spp. While dual-mycorrhizal associations are often shared among species within the same plant genus or family, there have been relatively few studies testing for differences in the extent of AM and EM colonization within individual dual-mycorrhizal plant lineages.

Some dual-mycorrhizal plants can engage in a phenomenon termed mycorrhizal switching in which they shift towards either AM or EM colonization in response to environmental or plant cues (Teste et al., 2020). Mycorrhizal switching can entail either the complete loss of one of these mycorrhizal guilds or a shift in colonization rates to the point that one mycorrhizal type becomes dominant (Teste et al., 2020). Mycorrhizal switching has been documented in response to topographic position, seasonal drought, soil age, habitat type, and spatial factors (Watson et al., 1990; Moyersoen & Fitter, 1999; Querejeta et al.,

2009; Albornoz et al., 2016; Van Nuland et al., 2023). Plant development has also been implicated as a trigger for mycorrhizal switching on some dual-mycorrhizal hosts, with a frequently observed transition from high AM colonization in young plants, towards increasing EM colonization as plants age (Chilvers et al., 1987; Chen et al., 2000; Dickie et al., 2001; Santos et al., 2001). Teste et al. (2020) proposed the classification of such plants as "temporally dependent dual-mycorrhizal hosts". Preferential association with AM fungi at the seedling stage is hypothesized to facilitate seedling establishment (Teste et al., 2020). Although some temporally dependent dual-mycorrhizal hosts retain low levels of AM colonization throughout development, in extreme cases hosts can completely lose AM colonization and become entirely EM after approximately one year (de Mendonça Bellei et al., 1992). Although studies of dual-mycorrhizal plants have hinted at variability in the degree of temporal-dependence, there have been few systematic studies comparing successional trajectories on closely related hosts.

In this study, we focus on the dual-mycorrhizal tree genus *Populus* to test the effects of plant host species identity and plant age on AM and EM colonization. Trees in the genus *Populus* have been widely used in plant-microbe interactions research and have been proposed as model dual-mycorrhizal systems (Cregger et al., 2021; Karst et al., 2021). Although there have been studies documenting variation in AM and EM colonization in *Populus* in response to tree genotype, temperature, soil moisture, soil depth, seasonality, heavy metal contamination, and leaf litter extract application (Lodge, 1989; Neville et al., 2002; Gehring et al., 2006; Piotrowski et al., 2008; Karliński et al., 2010; Van Nuland et al., 2023; Nash et al., 2025), there has been surprisingly little work examining both AM and EM colonization throughout plant development and it is unclear if *Populus* species should be classified as temporally dependent dual-mycorrhizal hosts (Dominik, 1958; Fracchia et al., 2021). Although the composition of the fungal and bacterial microbiome of *Populus* is known to change with plant age (Fracchia et al., 2021; Xie et al., 2023; Argiroff et al., 2024), only a few studies have tracked changes in AM and EM colonization through plant development on *Populus* (Dominik, 1958; Dove et al., 2021; Fracchia et al., 2024). Thus, it is not known whether *Populus* species undergo a shift in dominance of AM fungi to EM fungi as plants

age as has been demonstrated on other temporally dependent dual-mycorrhizal species (Teste et al., 2020). There are many studies demonstrating AM colonization on mature *Populus* trees of various species, so it is unlikely that they completely lose AM colonization with age, but it is possible that there is a predictable age-related decline in AM colonization rates (Lodge & Wentworth, 1990; Karliński et al., 2010; Bainard et al., 2011; Frymark-Szymkowiak et al., 2023; Van Nuland et al., 2023). The *Populus* genus contains around 30 species, distributed across six sections, which are all presumably dual-mycorrhizal (Eckenwalder, 1996; Wang et al., 2020). These species have unique biogeographic distributions and occupy a range of habitats, including boreal forest, semi-arid shrublands, mixed hardwood forests, and riparian zones. A DNA metabarcoding and microscopy-based field survey of five species of *Populus* in North America found a gradient of EM and AM colonization levels with *P. angustifolia* and *P. tremuloides* having the highest EM colonization and *P. deltoides* having the highest AM colonization, although *P. tremuloides* had the highest AM fungal richness (Van Nuland et al., 2023). Our study makes use of a pot culture approach using a field-sourced soil inoculum with plants maintained in a controlled growth chamber to isolate the effect of plant species identity on mycorrhizal guild abundance during the first year of growth on *P. tremuloides* and *P. trichocarpa*. We used metabarcoding and root staining/microscopy to profile fungal colonization on both *Populus* spp. at 5 and 12 months of plant growth. We used RNA metabarcoding instead of DNA based methods, as we have previously found it to recover AM sequences at much higher levels (Nash et al., 2025). We hypothesized that 1) both *Populus* hosts will be temporally dependent dual-mycorrhizal species, with a shift in predominance of AM colonization towards EM colonization as plants age, 2) *P. tremuloides* will display a preference for EM over AM colonization compared to *P. trichocarpa* which will have greater AM colonization, and 3) in addition to guild-wide patterns, there will be consistently early and late colonizing species.

Methods

Overview of methods

We inoculated 20 *P. tremuloides* and 20 *P. trichocarpa* seedlings with field soils sampled from *P. tremuloides* stands in southern Utah, along with four uninoculated controls of each species and grew them in pots for 12 months in a controlled growth chamber at Duke University's Phytotron facility. Fine roots were collected from each plant at 5 and 12 months and used for ink-vinegar staining and LSU RNA metabarcoding to measure colonization levels of AM, EM, and endophytic fungi as well as sequence abundance of individual taxa.

Inoculum collection and plant growth conditions

In September 2019, we collected 20 soil samples from the top 15 cm of soil in three *P. tremuloides* stands in Fishlake National Forest, Utah, United States that were spaced across 56 hectares. Soil samples were shipped back to Duke University and kept at 4°C for four months until inoculation. One month prior to inoculation, *P. tremuloides* and *P. trichocarpa* seeds (sourced by Sheffield's Seed, Locke, NY, USA from natural populations in Utah and Idaho, respectively) were sown into nursery trays with autoclaved vermiculite and grown covered under fluorescent lights until inoculation. A sterile potting mix was prepared by mixing vermiculite, perlite, peat, and sand at a 2:2:1:1 ratio and autoclaving twice at 121°C for two hours with two days in between each autoclave cycle. We inoculated one seedling of each plant host with each of the 20 soil inocula at a ratio of 1 part soil inoculum to 2 parts sterile potting mix to maintain biological replication and capture the natural heterogeneity of soil inocula. For some soil samples, we had to decrease this ratio because of a low quantity of inoculum, though inoculum levels were always the same between the two hosts to avoid systemic bias and we found no impact of inoculum levels on alpha or beta diversity metrics. Seedlings were planted into 7 cm diameter by 25 cm deep conical tree pots until 6 months of age at which point they were transplanted into 13 cm by 13 cm by 30 cm square tree pots filled with the sterile potting mix but no additional inoculum to prevent root binding. Plants were grown in a controlled growth chamber with a 16 h day/8 h night light cycle with a light intensity of approximately 400 umol m^{-2} s^{-1}, 60% humidity, 23° C daytime temperature, and 18° C nighttime temperature. Plants were fertilized throughout the experiment by watering pots with half

strength Hoagland's solution until saturation once per week. Plant height was collected at the time of sampling (see following section) at both 5 and 12 months by measuring the distance from the soil line to the apical bud.

Sample collection and molecular methods

At 5 and 12 months of plant growth, seedlings were gently extricated from pots and fine root samples were clipped from plants non-destructively with dissecting scissors and a scalpel to allow the same plants to be resampled. Fine roots with tightly adhering soil were flash frozen in liquid nitrogen immediately on collection and stored at -80°C until RNA extraction. A separate subsample of fine roots was rinsed of all adhering soil and stored in 10% KOH for staining and microscopic colonization scoring. For RNA extraction, approximately 1 gram of root tissue was cryogenically ground with a mortar and pestle and liquid nitrogen. RNA was extracted from ground roots using the Qiagen RNeasy PowerSoil Total RNA Kit (Qiagen, Carlsbad, CA, USA), followed by DNase treatment with TURBO DNase (Thermo Fisher Scientific, Waltham, USA), and then by a column cleanup with the Qiagen RNeasy PowerClean Pro Kit. RNA samples were tested for DNA contamination by amplification of the LSU gene with the primers LR0R and LR3 primers (Vilgalys & Hester, 1990) and those that had DNA contamination were subjected to additional DNase treatments until they failed to amplify. RNA samples were reverse transcribed into cDNA using the reverse primer LR5 (Vilgalys & Hester, 1990) and the Qiagen Omniscript RT Kit. cDNA was amplified using a two-step PCR barcoding approach with the primers LR0R and LR5 and the Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA). Amplicons were submitted to the Duke Sequencing Center for library preparation, pooling, and sequencing on a PacBio Sequel.

Root staining and colonization scoring

The fine root samples that were collected and preserved in 10% KOH were used to quantify the degree of root colonization by EM, AM, and endophytic fungi using the ink-vinegar staining method

(Vierheilig et al., 1998) followed by scoring of colonization using the magnified intersections method (McGonigle et al., 1990). For staining, we extended the KOH clearing step to 1 to 2 hours (judged by visually checking pigmentation of roots) because of high levels of pigmentation in the roots. After destaining in dilute vinegar, roots were cut into 1 cm segments and mounted in vinegar on a microscope slide with a total of approximately 20 cm of roots examined per plant. The roots were scored for a minimum of 100 root intersections. Each root intersection was scored using the following categories: ectomycorrhizal root tip, AM vesicles, AM hyphal coils, arbuscules, AM hyphae, microsclerotia, endophytic hypha, or uncolonized. AM hyphae were differentiated from endophytic hyphae by their lack of septa, greater diameter, and when possible their connection to vesicles, coils, or arbuscules. We acknowledge that other early-diverging fungal groups have similar hyphal morphology, but these groups were not common in our metabarcoding dataset and we have high confidence in our scoring of AM hyphae. Total AM colonization was calculated by summing together vesicle, hyphal coil, arbuscule, and AM hyphal colonization. Total endophytic colonization was calculated by summing together microsclerotia and endophytic hyphal colonization.

Sequence Analysis and Statistics

PacBio LSU sequencing data were analyzed in QIIME2 version 2024.2.0 (Bolyen et al., 2019). CCS sequences were denoised, trimmed of primers, and chimera-checked with Dada2 (Callahan et al., 2016). Taxonomy was assigned to ASV (Amplicon sequence variant) sequences using the classify-consensus-blast command in QIIME2 (Bokulich et al., 2018) against a database of NCBI eukaryotic LSU sequences. Taxonomic assignments were reviewed for the 200 most abundant ASVs and other seemingly erroneous taxonomic assignments (notably for ASVs mistakenly identified as *Terfezia* and *Alnicola*) by manually examining BLAST results and adjusting taxonomic assignments when necessary (see Supplemental File S1 for taxonomy edits). Sequence data were rarefied to 816 sequences per sample and samples with fewer sequences than this were excluded from further analysis. The diversity of most samples had saturated at this rarefaction depth. Fungal ASVs were assigned to guilds based on the

FUNGuild database (Nguyen et al., 2016), with the primary guild used in cases where an ASV was assigned multiple potential guilds. We also conducted manual annotation of known endophytes (see Supplemental File S1). We conducted Sanger sequencing of 65 visibly colonized EM root tips by first extracting in a solution of Tris, EDTA, and KCl ph 9.5, followed by amplification with the primers 5.8SR and LR3, and submission for Sanger sequencing by Eurofins USA.

We tested for differences in guild colonization using both microscopic colonization scoring data and sequencing data with repeated measure mixed models including a random effect of plant individual in the *lme4* package in R (Bates et al., 2015) with square root transformations of response variables to meet assumptions of normality. For the repeated measure models that were significant ($P < 0.05$), we performed post-hoc ANOVA tests for the effect of species with the dataset split by plant age. We found no effect of the stand from which soil inocula originated on fungal guild abundance and thus we omitted stand from final models, considering each soil core as an independent replicate. We identified individual fungal genera that varied between conditions (*Populus* species or plant age) by 1) first identifying genera that were entirely absent from one condition and present in at least 5 samples in the other condition and 2) running ANCOMBC-2 tests with false discovery rate p-value corrections to identify differences in sequence abundance of remaining genera – genera were considered differentially abundance if they either met the presence/absence filter or had a significant ANCOMBC-2 result. Data were visualized using the *ggplot2* package (Wickham & Sievert, 2009) in R (R Core Team, 2013).

Results

Overview of fungal communities

We detected 742 ASVs (Amplicon sequence variants) that were distributed across 73 fungal genera with LSU RNA metabarcoding. The root-associating fungal communities had generally low diversity and showed a decrease in diversity through time from 29.6 ± 10.4 ASVs at 5 months of growth down to 14.9 ± 4.7 ASVs at 12 months of growth ($P < 0.0001$). Fungal richness was slightly higher on *P.*

trichocarpa than on *P. tremuloides* at both timepoints ($P = 0.04$). Many of the most abundant ASVs were from known *Populus* endophytes including *Hyaloscypha* spp. and *Ilyonectria* spp (Bonito et al., 2016). The most abundant EM ASVs were *Hebeloma* spp., *Tuber* spp., *Sphaerosporella* spp., and *Cenococcum geophilum*. We also observed high abundance of the biotrophic genera *Ceratobasidium*, *Serendipita*, *Flagelloscypha*, and *Tulasnella*, though it is unclear what type of symbiotic interaction these taxa form with *Populus*. Distinct EM morphotypes were observed on plant roots that could be identified in some cases by Sanger sequencing (see Fig. 1a, 1b). We also documented fungal endophytic structures such as microsclerotia, although we are unable to link specific endophytic structures with taxonomic information (see Fig. 1c). We documented AM colonization based on the presence of AM hyphae, arbuscules, vesicles, and hyphal coils (see Fig. 1 d-f). Control plants were generally free of substantial visible colonization, although modest levels of endophytic colonization were noted in some pots. Metabarcoding showed that *Hyaloscypha*, *Ilyonectria*, *Solicoccozyma*, *Ceratobasidium*, *Tuber*, *Penicillium*, and *Trichoderma* were the most common contaminants of control plants (>2% mean abundance), although most of these taxa are known inhabitants of *Populus* roots and it is possible that they were present in the initial soil inoculum and later moved between pots.

Patterns in guild abundance

EM colonization on both *Populus* hosts increased with plant age (confirmed by microscopy and metabarcoding), while endophytic colonization decreased with age (confirmed by microscopy but not metabarcoding; see Fig. 2). Based on microscopy, the rate of EM fungal recruitment seemed to be faster for *P. tremuloides* than *P. trichocarpa*, with greater EM colonization on *P. tremuloides* than *P. trichocarpa* at 5 months ($P = 0.008$), but equal EM colonization between hosts by the 12 month timepoint (although this pattern was not confirmed by LSU metabarcoding which found similar EM colonization levels between the two species regardless of plant age). The two hosts had similar endophytic colonization at 5 months, but at 12 months *P. tremuloides* had higher endophytic colonization than *P. trichocarpa* based on microscopy ($P = 0.025$), but similar endophytic colonization based on sequencing

($P = 0.19$; see Fig. 2). The vast majority (>90%) of the endophytic sequence abundance was attributable to dark septate endophytic fungi in the order Helotiales. Among taxa in the Helotiales, *Hyaloscypha* was the most abundant. *P. trichocarpa* had substantial AM colonization based on microscopy (24% ± 29%; see Fig. 2a), but modest sequence abundances (3.6 % ± 5.3%; see Fig. 2b). *P. tremuloides* was entirely devoid of AM colonization (confirmed by both sequencing and microscopy), while on *P. trichocarpa*, AM fungi successfully colonized and increased in sequence abundance and colonization rate with plant age (see Fig. 2). Most of the AM colonization on *P. trichocarpa* was attributable to hyphal colonization (13.2% ± 15.6% root length colonized), while vesicles (8% ± 15.3%), arbuscules (2.4% ± 3.3%), and hyphal coils (0.49% ± 0.81%) were observed at lower rates. On *P. trichocarpa*, 41% of plants were dual-mycorrhizal, 35% of plants were entirely AM, 21% were entirely EM, and 3% were non-mycorrhizal based on microscopic colonization scoring averaged across both plant ages. Dual-mycorrhizal *P. trichocarpa* plants had a weak tendency to be taller than entirely AM plants at 12 months plant age, but the effect was not significant ($P = 0.097$).

Early and late colonizing genera

The genera *Mycena*, *Diaporthe*, *Polyphilus*, *Solicoccozyma*, *Trichocladium*, *Cenococcum*, *Aquilomyces*, *Exophiala*, and *Tulasnella* were frequently associated with 5 month old plants, but then declined in abundance or disappeared entirely on the 12 month old plants (see Fig. 3). In the case of *Cenococcum*, this shift was readily observable during microscopic examination of the roots due to their easily identifiable black ectomycorrhizae, although we did not microscopically score *Cenococcum* abundance specifically (personal observation, see Fig. 1b). The only genus that increased in abundance with plant age was *Sphaerosporella*, but this may have been an airborne contaminant because it is a common environmental contaminant and was observed fruiting in one pot before quickly spreading to other pots shortly before the 12 month sampling timepoint. This was confirmed by Sanger sequencing of fruiting bodies and root tips in inoculated plants and the presence of *Sphaerosporella* on two out of eight uninoculated control plants at an average of 13% sequence abundance. The AM fungal genera *Glomus*

and *Rhizophagus* occurred on *P. trichocarpa* but not on *P. tremuloides* and were the only fungal genera that displayed plant host preference. Both *Glomus* and *Rhizophagus* seemed to increase in abundance with plant age on *P. trichocarpa* (see Fig. 3), but this pattern was not significant.

Discussion

We hypothesized that 1) both *Populus* spp. would behave as temporally dependent dual-mycorrhizal species, exhibiting a shift in predominance of AM to EM colonization during the first year of growth, 2) *P. tremuloides* would have greater EM colonization than *P. trichocarpa*, while *P. trichocarpa* would have greater AM colonization and 3) there would be distinct cohorts of early and late colonizing fungal groups. We found little support for hypothesis 1, instead finding that both guilds of mycorrhizal fungi increased in tandem on *P. trichocarpa*, while *P. tremuloides* was not colonized by AM fungi at any point, but did exhibit an age-dependent increase in EM colonization. We found mixed support for hypothesis 2, with greater EM colonization on *P. tremuloides* than *P. trichocarpa* at 5 months plant age that became similar by the 12 month timepoint and consistent AM colonization on *P. trichocarpa*, but a complete lack of AM colonization on *P. tremuloides*. We found strong support for the existence of an early colonizing community of fungal symbionts, but little evidence for a distinct late colonizing community (hypothesis 3). Our findings suggest that both EM and AM colonization may continue increasing beyond the first year of growth in the two *Populus* spp. examined and it is not known if or when a decline in AM colonization would occur. Our approach also allowed us to document changes in endophytic colonization, which decreased with plant age. Studies on other plant hosts have found that mycorrhizal switching can occur on timescales ranging from 3 to 16 months (Chen et al., 2000; Dickie et al., 2001; Santos et al., 2001), while an early study of European *Populus* spp. suggests that AM colonization can be maintained during the first two years of growth (Dominik, 1958). A time-series study of the first 50 days of growth in *Populus tremula* x *alba* cuttings in pot culture found a steady increase in both EM and endophytic colonization (Fracchia et al., 2021), while another study in a *Populus* plantation found an increase in EM colonization during the first two years of growth (Argiroff et al., 2024). Collectively, these results show that

296 colonization by AM, EM, and endophytic fungi is dynamic through time and these different guilds may
297 peak in abundance at different periods of plant development. A number of studies demonstrating AM
298 colonization on mature *Populus* species demonstrates that even if temporally-dependent mycorrhizal
299 switching does occur, it likely entails shifts in colonization rates rather than the absolute loss of AM
300 colonization (Lodge & Wentworth, 1990; Karliński et al., 2010; Bainard et al., 2011; Frymark-
301 Szymkowiak et al., 2023; Van Nuland et al., 2023).

302 In addition to the temporal patterns in guild abundance, we also identified specific early-colonizing
303 fungal genera that declined in abundance or disappeared entirely from the root system by 12 months. This
304 group included the genera *Mycena*, *Diaporthe*, *Polyphilus*, *Solicoccozyma*, *Trichocladium*, *Cenococcum*,
305 *Aquilomyces*, *Exophiala*, and *Tulasnella*. The EM fungus *Cenococcum* has been previously documented
306 as an early fungal colonizer on *Populus* (Dominik, 1958), although others have found extensive
307 colonization by *Cenococcum* in mature stands (Vélez et al., 2021; Nash et al., 2025). This group of early-
308 colonizing fungi includes taxa that are notable for having high saprotrophic capabilities, despite their
309 lifestyles as plant biotrophs. *Tulasnella* is a genus of orchid mycorrhizal fungi and endophytes that has
310 strong plant cell degradative capabilities (Adamo et al., 2020). *Mycena* spp. have been classically
311 considered to be a free-living saprotrophs, but recently have been documented as potential endophytes in
312 healthy living roots and some species may transfer nutrients to plants (Thoen et al., 2020; Harder et al.,
313 2023). However, our metabarcoding approach may have detected high levels of sequences from *Mycena*
314 and other soil-dwelling saprotrophs because rhizosphere soil was included in our sample collection.
315 *Trichocladium*, *Exophiala*, *Polyphilus* and *Aquilomyces* are all dark septate endophytic genera (Knapp et
316 al., 2015; Ashrafi et al., 2018) which have also been linked to high saprotrophic capabilities (Caldwell et
317 al., 2000). Our findings suggest that this saprotrophic capability may be a key trait enabling fungal
318 symbionts to quickly colonize plants, but may not be associated with long-term persistence of fungal
319 symbionts as plants age. Fracchia et al. (2021) found similar results in a time-series study of *Populus* on a
320 much shorter timescale, with saprotrophic fungi being very abundant on roots in the first few days of

growth. Dark septate endophytes are understudied, but some experimental work shows that they can transfer nutrients to plants (Almario et al., 2017; Xu et al., 2020) and thus may represent an important nutrient acquisition symbiosis during early plant growth. In this study, the dark septate endophytic genus *Hyaloscypha* was found to be one of the most prolific colonizers on both *Populus* spp. throughout the growth period, demonstrating that certain dark septate endophytic taxa can persist for longer as plants age. Past studies have identified both *Hyaloscypha* and *Exophiala* as being core members of the *Populus* root fungal community (Shakya et al., 2013; Nash et al., 2025). A past culture-based study of *Populus* root fungal symbionts found that endophytes were among the most common culturable members of root communities (Bonito et al., 2016), with frequent isolations of *Ilyonectria*, *Hyaloscypha*, and *Exophiala* which were abundant in our dataset. Despite the growing body of work showing that dark septate endophytes are prolific colonizers on dual-mycorrhizal hosts (including *Populus*), many mycorrhizal studies still overlook the role of this guild in studies of plant nutrition and stress tolerance. Dark septate endophytes are prevalent in forests, grasslands, and shrublands and have been implicated in biotic interactions and modulation of soil biogeochemical cycling (Netherway & Bahram, 2024; Netherway et al., 2024). The temporal dynamics and potential biotic interactions of fungal endophytic colonization on root systems that are co-colonized by AM and EM fungi remains an underexplored area of research.

Fungal alpha diversity decreased by half from 5 months to 12 months of plant growth. The pot culture method that we employed did not allow for recruitment of new fungal symbionts by dispersal and is thus somewhat unrealistic compared to natural conditions where continuous dispersal allows for recruitment of new symbionts over the lifespan of a tree. However, this time-dependent decrease in alpha diversity suggests that in the absence of dispersal, root associated fungal communities experience a loss of diversity as plants age. Mycorrhizal fungal diversity is thought to promote plant growth and higher rates of nutrient transfer to plants (Nash et al., 2020; Anthony, 2025) and it is unknown how the observed decrease in fungal diversity with plant development affected the quality of mycorrhizal benefits provided to the plant hosts.

For our study, we grew trees in natural soil inocula under controlled growth chamber conditions to isolate the role of plant host identity on structuring root-associated fungal communities without the confounding influence of environmental variation. The two *Populus* spp. examined exhibited distinct preferences for AM and EM colonization, suggesting that they exist along a spectrum of dual-mycorrhizal colonization. *P. trichocarpa* displayed moderate levels of AM colonization, while *P. tremuloides* showed very little evidence for AM colonization. *P. tremuloides* had higher early rates of EM colonization than *P. trichocarpa* based on microscopy, although the effect of plant species on EM colonization became nonsignificant as the plants aged. The findings regarding AM colonization of *P. tremuloides* are consistent with other studies showing that the species is a poor AM host (Kaldorf et al., 2002; Karst et al., 2021). However, a microscopy-based study on mature *P. tremuloides* found an average AM colonization rate of 6% (Neville et al., 2002) and another molecular-based study found modest AM association with *P. tremuloides* (Nash et al., 2025). Interestingly, *P. tremuloides* x *P. tremula* hybrids have been shown to exhibit greatly decreased AM colonization in response to nitrogen fertilization, while AM colonization of *P. trichocarpa* was unaffected and so the usage of an inorganic fertilizer in our experiment may have inhibited AM colonization of *P.* tremuloides, while allowing for AM colonization of *P. trichocarpa* due to host-dependent differences in AM sensitivity to inorganic nutrient application (Baum & Makeschin, 2000). Thus, *P. tremuloides* should still be assumed to be dual-mycorrhizal despite the lack of evidence for AM colonization in this study. The higher rate of EM colonization on *P. tremuloides* than *P. trichocarpa* that we found is consistent with a continental-scale comparison of five *Populus* species (Van Nuland et al., 2023). These findings suggest that previously observed differences in mycorrhizal colonization among *Populus* spp. (Van Nuland et al., 2023) are not solely the result of environmental variation and fungal biogeography, but are also due to differences in host preference for AM and EM colonization. Although we found stark host-dependent differences in mycorrhizal colonization between the two *Populus* spp., we found no individual fungal taxa that differed in abundance between the two hosts outside of the AM genera *Rhizophagus* and *Glomus*. It is possible that *P. trichocarpa*-specific fungal symbionts were excluded from our study because we sourced soil inocula from a *P. tremuloides*

372 stand outside of the natural range of *P. trichocarpa*. Additional controlled experiments that use a wider
373 phylogenetic diversity of *Populus* species grown with diverse soil inocula are needed to advance our
374 knowledge of fungal host-specificity in the *Populus* genus.

375 Our experiment made use of a simplified growth chamber environment to isolate the effects of hosts
376 species identity and plant age on root fungal colonization, but there are still many unresolved questions
377 about the impact of environmental context on determining dual-mycorrhizal colonization during early
378 plant development and the mechanisms that underlie mycorrhizal switching. The density and proximity of
379 adult mycorrhizal hosts may influence colonization dynamics of seedlings due to hyphal spread (Dickie et
380 al., 2001). Soil moisture levels can shift the balance between AM and EM fungi and may alter the rate of
381 microbiome development (Lodge, 1989; Xu et al., 2018). The long-term trajectories of dual-mycorrhizal
382 colonization is also likely influenced by the ability of mycorrhizal symbionts to persist during winter
383 when plant carbon availability is low and fungal symbionts may experience freezing. EM fungi are more
384 resistant to soil freezing than AM fungi and successive freeze-thaw cycles may result in a decline in AM
385 colonization over time on dual-mycorrhizal hosts (Kilpeläinen et al., 2016). Additionally, Lodge and
386 Wentworth (1990) have documented potential antagonism between EM and AM fungi in the same root
387 system, which could result in transitions between these guilds if one of them gains a competitive
388 advantage. In our study, AM and EM colonization increased in tandem as plants aged while endophytic
389 colonization decreased, hinting at a possible unexplored role of competition with endophytic fungi in
390 structuring *Populus* root-associated fungal communities. Changes in host metabolism through plant
391 development are likely also strong controls on fungal community composition (Xie et al., 2023; Fracchia
392 et al., 2024). Determining how the interactions of these various environmental and host factors structure
393 root fungal communities on dual-mycorrhizal hosts presents a large challenge for future researchers.

394 To conclude, our study demonstrates that plant age-dependent changes in root fungal community
395 composition in *Populus* are distinct from patterns observed in other dual-mycorrhizal hosts and
396 demonstrates that there is variation in association with AM fungi among different *Populus* spp. We show

that saprotrophic capabilities may be an important trait of early colonizing fungal endophytes. Our findings indicate the potential for diverse developmental trajectories in dual-mycorrhizal hosts and stress the importance of examining endophytic fungal communities in addition to mycorrhizal fungi. Developmentally induced shifts in root-associated fungal communities likely have functional implications for rates of nutrient transfer and ecosystem processes that provide an exciting avenue for future research.

Data Availability

Code underlying the analyses presented in this paper is available at https://github.com/jakenash12/PopulusSuccession. All data underlying the analyses presented in this paper are included in Supplemental File S1. Representative sequences for OTUs sequenced for this project will be made available on GenBank upon acceptance of this paper for permanent data deposition.

Figure Captions

Figure 1. Morphology of fungal colonization observed throughout the experiment with approximate scale bars. (a) an ectomycorrhiza of *Tuber anniae*, (b) abundant ectomycorrhizae of *Cenococcum geophilum* observed on 5 month old plants, (c) endophytic microsclerotia, (d) arbuscular mycorrhizal arbuscules, (e) arbuscular mycorrhizal vesicles, (f) arbuscular mycorrhizal hyphal coils

Figure 2. Stacked bar plots indicating the (a) relative root colonization and (b) relative sequence abundance of arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and endophytic fungi. For the microscopic colonization scoring data, the uncolonized category refers to root intersections that were devoid of visible fungal colonization. For the sequencing dataset, fungi that were not annotated as plant symbiotic taxa were grouped into the "other" category and primarily includes saprotrophs and plant pathogense . (c) Significance of difference in guild abundance by plant age and species identity determined my repeated measures mixed models is displayed. Asterisks in the top left corner of each box indicate significance determined by microscopy and those in the lower right corner indicate significance determined by LSU RNA metabarcoding. The dagger symbol † indicates the effect of plant species was marginally significant ($P < 0.10$) for EM microscopic colonization, with post-hoc tests finding a strongly significant effect of plant species at 5 months plant age ($P = 0.008$), but no effect at 12 months plant age.

Figure 3. A heatmap showing the abundance of fungal genera that differed either by plant age or by time as well as the top five fungal genera that did not vary by plant species or age. Columns represent individual samples and tiles in white indicate the absence of that genus in a sample. Sequence abundance of each genus was scaled between 0 and 1 to allow for convenient visualization.

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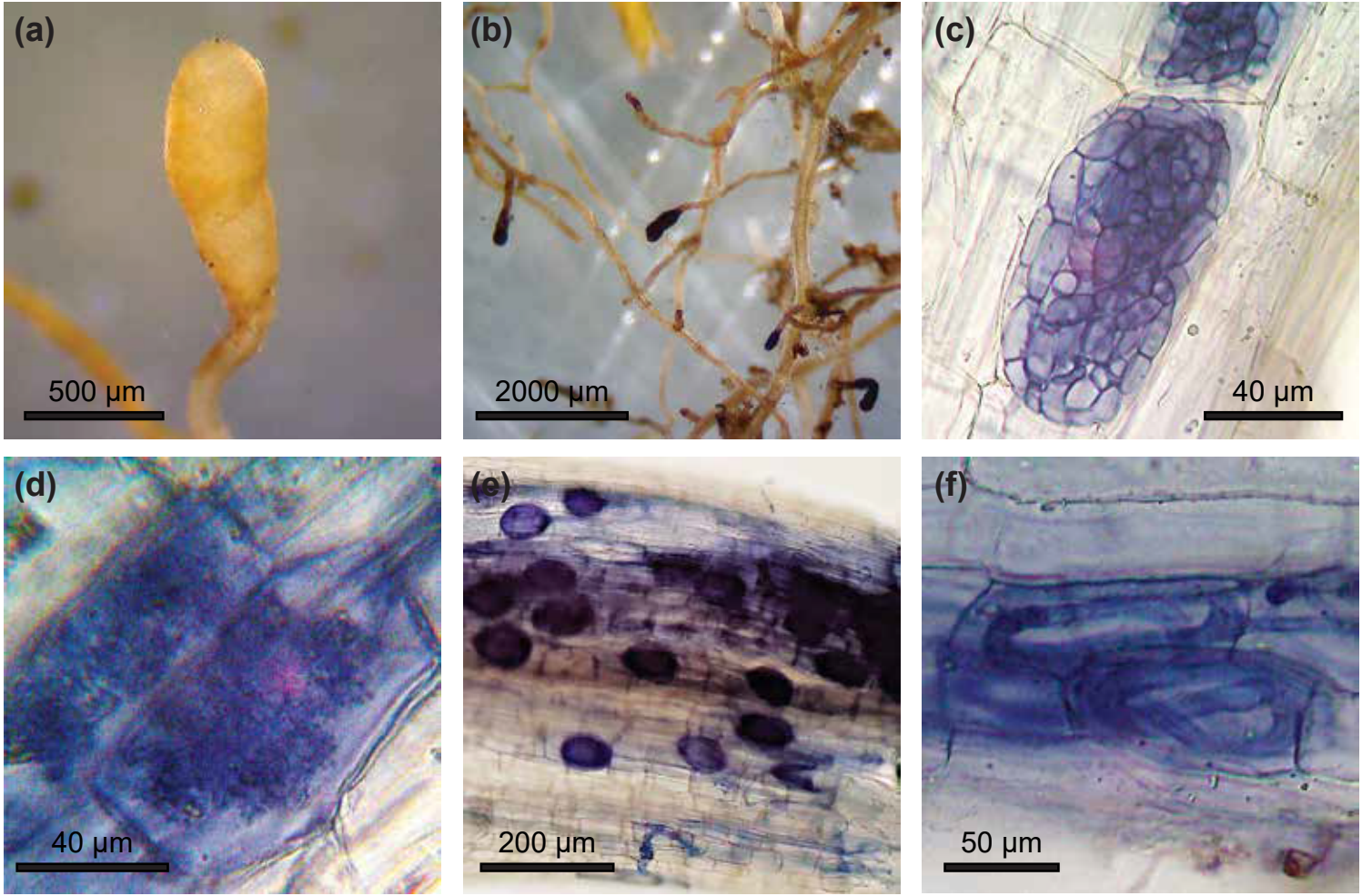
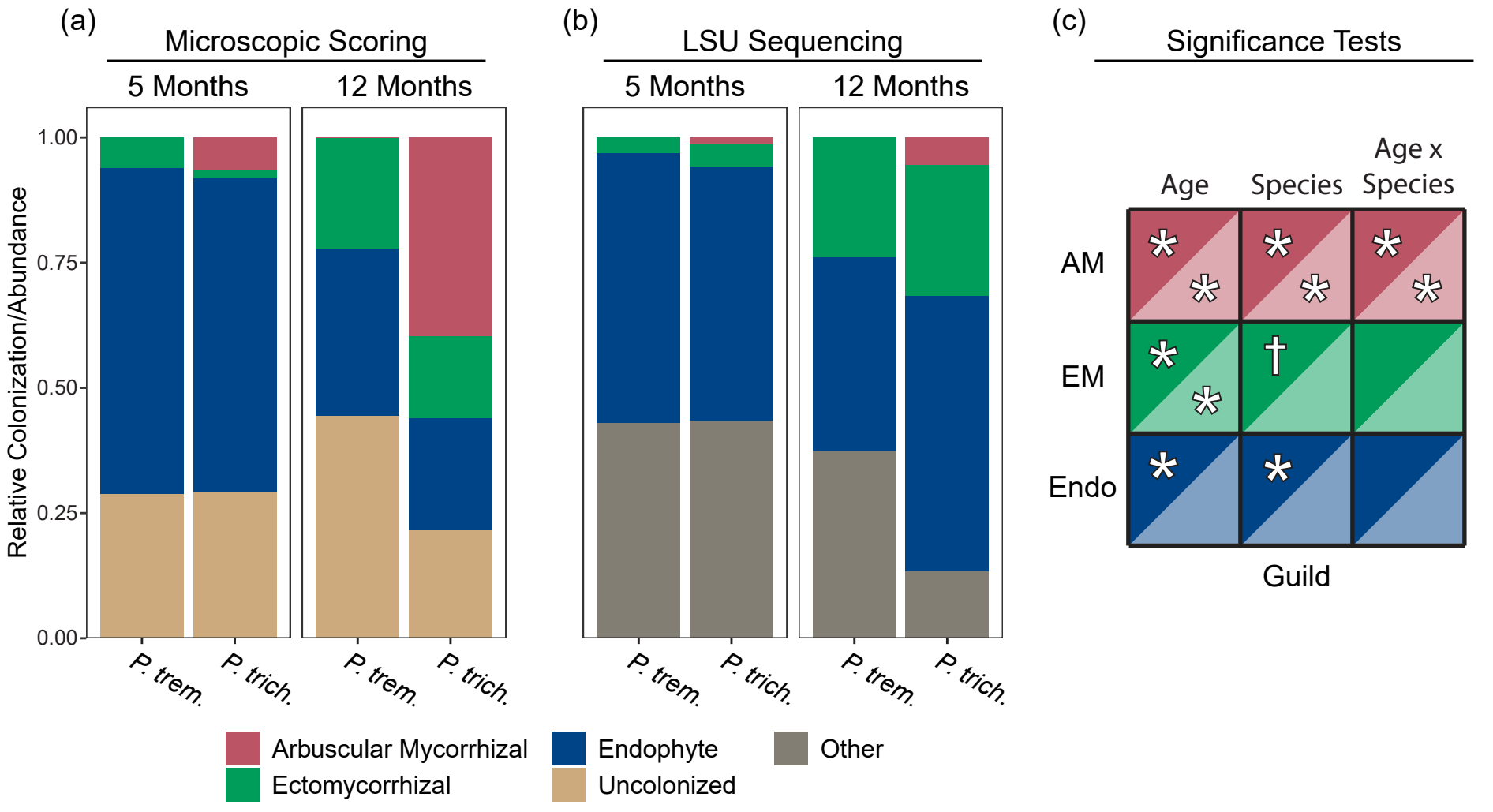

(a)
500 µm
(b)
2000 µm
(c)
40 µm
(d)
40 µm
(e)
200 µm
(f)
50 µm


(a)
Microscopic Scoring
5 Months
12 Months
Relative Colonization/Abundance
1.00
0.75
0.50
0.25
0.00
P. trem.
P. trich.
(b)
LSU Sequencing
(c)
Significance Tests
Age
Species
Age x Species
AM
EM
Endo
Guild
Arbuscular Mycorrhizal
Ectomycorrhizal
Endophyte
Uncolonized
Other