

# Arbuscular mycorrhizal fungal communities change among three stages of primary sand dune succession but do not alter plant growth

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Plant interactions with soil biota could have a significant impact on plant successional trajectory by benefiting plants in a particular successional stage over others. The influence of soil mutualists such as mycorrhizal fungi is thought to be an important feedback component, yet they have shown benefits to both early and late successional plants that could either retard or accelerate succession. Here we first determine if arbuscular mycorrhizal (AM) fungi differ among three stages of primary sand dune succession and then if they alter growth of plants from particular successional stages. We isolated AM fungal inoculum from early, intermediate or late stages of a primary dune succession and compared them using cloning and sequencing. We then grew eight plant species that dominate within each of these successional stages with each AM fungal inoculum. We measured fungal growth to assess potential AM functional differences and plant growth to determine if AM fungi positively or negatively affect plants. AM fungi isolated from early succession were more phylogenetically diverse relative to intermediate and late succession while late successional fungi consistently produced more soil hyphae and arbuscules. Despite these differences, inocula from different successional stages had similar effects on the growth of all plant species. Host plant biomass was not affected by mycorrhizal inoculation relative to un-inoculated controls. Although mycorrhizal communities differ among primary dune successional stages and formed different fungal structures, these differences did not directly affect the growth of plants from different dune successional stages in our experiment and therefore may be less likely to directly contribute to plant succession in sand dunes.

Soil organisms can strongly influence plant succession, the changes in plant communities over time, which is a foundational process for terrestrial ecosystem development. Soil biota alter plant community structure by facilitating nutrient mobilization and uptake (Vitousek et al. 1987, Smith and Read 2008), altering competition between plants (Fitter 1977) pathogenesis (Olf et al. 2000, Klironomos 2002, Petermann et al. 2008), and through direct competition with plants (Diaz et al. 1993, Alberton et al. 2007). Therefore soil organisms can influence established mechanisms of plant succession such as competition for nutrients (Clements 1916, Connell and Slatyer 1977, Tilman 1985) and also represent a novel determinant of plant succession (De Deyn et al. 2003, van der Putten et al. 2009).

Soil biotic effects on plant succession usually operate through feedbacks whose outcome depends on the specific soil biota and mechanism of their effect. Feedback effects occur through reciprocal changes in plant and soil biotic communities and are generally negative when plants are grown in ‘home’ soils (Kulmatiski et al. 2008). In secondary succession of old fields, negative feedback from soil pathogens and parasites limit the growth of early

succession plants over those in later succession (De Deyn et al. 2003, Kardol et al. 2006, 2007). Positive soil feedback on late succession plant growth was attributed to the accumulation of soil mutualists such as mycorrhizal fungi (Kardol et al. 2006). Parallel work in primary succession on sand dunes also shows that soil pathogens can limit early succession plants (van der Putten et al. 1993) but mutualisms with arbuscular mycorrhizal (AM) fungi offset these effects (Little and Maun 1996). AM fungi can also facilitate phosphorus uptake that may benefit plants in both late primary and secondary succession when phosphorus is more limiting (Janos 1980, Vitousek and Farrington 1997, Lichter 1998a). AM fungi therefore may either accelerate or retard succession depending on the specific function they provide.

Particular phylogenetic lineages of AM fungi and fungal morphologies are more effective at specific functions (Maherali and Klironomos 2007, Powell et al. 2009), therefore specific fungal shifts driven by plant feedback could affect plant succession in predictable ways. Plant hosts and fungal symbionts can preferentially allocate resources to partners that provide a needed function resulting in positive feedbacks on plant and fungal growth (Kiers et al. 2011).

Therefore if pathogen protecting AM fungi form symbioses with early succession plants, it should result in a positive feedback, stabilizing plant–mycorrhizal interactions and retarding succession. However, the majority of plants tested so far appear to cultivate less beneficial AM fungi over time resulting in negative feedbacks (Bever 2002). Such negative feedbacks could accelerate succession by favouring newly dispersed plant species over established species.

Here we examine how AM fungi differ among primary successional stages and whether these fungal communities positively or negatively affect the growth of dominant plant species from each successional stage in a reciprocal transplant experiment of early, intermediate or late succession plants and fungi. We used molecular analysis to characterize AM propagules from each successional stage before growing plants with them. If AM fungi increase the growth of plant hosts from their own successional stage, this indicates positive feedback that could slow succession. In contrast, if AM fungi increase growth of plant hosts from other successional stages this would indicate negative feedback that may accelerate plant succession. After plants were grown with AM fungi, we examined whether AM structures and fungal growth of communities from each successional stage was affected by plant hosts. If plant hosts influence mycorrhizal fungal growth, then the number of fungal structures should differ among plant hosts. If soil nutrients and edaphic conditions influence fungi, then AM fungal growth and structures should reflect successional differences in the origin of each inoculum.

## Material and methods

To determine interactions between AM fungi and plants during succession, we collected soils from Wilderness State Park, Michigan, USA (45°43'N, 84°56'W), a previously described sand dune successional series (Lichter 1998b) where 102 ridges have formed at an average rate of approximately one dune every 32 years (Lichter 1995). Soils were collected during June 2007. We selected three pairs of dunes that represented distinctly different stages in both plant community composition and edaphic conditions including soil pH and soil nutrients (Lichter 1998b). The youngest dunes were 10 and 35 years old respectively, intermediate-aged dunes formed 235–295 years before present, and late successional dunes formed 450 and 845 years before present (Lichter 1997). On each dune we selected five random points along a 100 m transect established parallel to the shoreline at the apex of each dune. At each point, we collected ten soil cores using autoclaved aluminum coring cans and carefully transferred them into bags. Soils were refrigerated (4°C) within 24 h, transported back to the Univ. of Guelph, Ontario, Canada and held at 4°C.

## Seed collections and plant succession assignment

Within this dune succession, we chose to use plant species that were dominant in each successional stage (but not in others) to increase the likelihood that any mycorrhizal effects we observed could be applied broadly to plant succession.

We collected seeds from plant species across the series at Wilderness State Park over the summer of 2007. Dominant plant species for each successional stage were chosen using percent cover data along the successional series (Lichter 1998b) and personal observations (Supplementary material Appendix A2). For early successional species we used *Ammophila breviligulata* and *Artemisia campestris*. Both occur in the earliest dunes and are rarely present in dunes older than 100 years. For mid succession, we selected *Calamovilfa longifolia*, *Schizachyrium scoparium* and *Pinus strobus*. *Calamovilfa longifolia* replaces *A. breviligulata* as the dominant dune grass in early succession (< 100 years b.p.) but is still relatively abundant as *P. strobus* colonizes and forms a closed canopy forest (~225 years after formation). *Schizachyrium scoparium* becomes dominant at the last stages of open dunes, but is absent after the canopy closes (~250 years b.p.). For late succession we used *Pinus resinosa*, *Deschampsia flexuosa* and *Acer rubrum*. *Pinus resinosa* replaces *P. strobus* as the dominant canopy tree which, in turn is eventually replaced by *A. rubrum*. *Deschampsia flexuosa* is the only major dominant understory grass in late succession. In sites older than ~835 years there has been significant anthropogenic influence such as burning and logging to promote the growth of particular harvestable species (Lichter 1998b). For this reason they were excluded from our successional analysis.

Although these plant species dominate their respective successional stage, they also have important differences in life-history characteristics. Within this chronosequence, dominant grasses in early succession are replaced by tree species in intermediate and late succession. These species also differ strongly in their known mycorrhizal interactions, for example *Pinus* species associate with ecto-mycorrhizal species rather than AM fungi used here. These differences in plant traits may make it more likely to see differential effects of mycorrhizal inocula related to plant functional types rather than only successional origin. For individual feedback responses, one can compare plant species individually with mycorrhizal inocula from different successional stages rather than among plant species. Due to seed predation in the field and low germination success, we obtained seeds for dominant tree species (*Acer rubrum*, *Pinus resinosa*, *P. strobus*) from the Ontario Ministry of Natural Resources Tree Seed Facility which field collected seeds sources from a directly adjacent region in Canada. However, because these seeds were not collected within this successional series (local feedback) we conducted statistical analyses both with and without their inclusion.

## Inoculum preparation

To isolate AM fungal inoculum from each successional stage, we combined and homogenized 2.4 kg of soil from dunes of similar age. Each combined soil contained an equal amount of soil from five random points along each dune to pool spatial variation within dunes of the same age. We then took 600 g of soil from each of the combined soils (1.8 kg total) to establish a common mycorrhizal inoculum containing all possible AM fungi. We used repeated sucrose-centrifugation of soils (200 g isolation<sup>-1</sup>) to collect spores

and hyphae (Brundrett et al. 1994) for fungal inocula. Spores and hyphae were rinsed thoroughly with autoclaved, de-ionized water, sonicated for one minute to remove surface debris, rinsed again and then re-hydrated in 100 ml of autoclaved, de-ionized water (final concentration 18 g soil ml<sup>-1</sup> inoculum). To control for differences in microbial contaminants introduced with AM inoculum, we also collected a microbial filtrate by passing the initial spore filtrates through a 25 µm sieve. Microbial filtrates from all dunes were combined to represent a common microbial wash added as a control (Koide and Li 1989) to all AM inocula. AM fungal inoculum and microbial wash was maintained at 4°C for two weeks prior to plant inoculation. Final inocula were water only (hereafter 'Control'), microbial wash alone (hereafter 'Wash'), microbial wash + AM fungal inoculum from early successional dunes (hereafter 'Early'), microbial wash + AM fungal inoculum from intermediate age dunes (hereafter 'Intermediate'), microbial wash + AM fungal inoculum from late successional dunes (hereafter 'Late'), and microbial wash + combined AM fungal inocula from all stages (hereafter 'All').

### Molecular analysis of fungal inocula

We used DNA cloning and sequencing to characterize and compare the AM fungal inoculum of each successional stage that we used in the experiment. Ten ml of inoculum from each successional stage was used for each DNA extraction and analysis. Each sample was spun at 2500×g for 10 min and the supernatant removed. We extracted DNA from inocula (spores and hyphae) using a PowerMax Soil kit according to the manufacturer's instructions with an initial step where inocula were ground under liquid nitrogen. Four replicate PCR amplifications were run for each inoculum extract using the Glomeromycota specific primers, AML1 and AML2 (Lee et al. 2008) and the following PCR reaction mix: 20µl total reaction volume; final concentrations: 1× PCR buffer; 1.5 mM MgCl<sub>2</sub>; 0.2 mM dNTP's; 0.5 µM of each primer and 0.75 U of Platinum *Taq* Polymerase. Thermocycling parameters were slightly altered from those published in Lee et al. (2008): 94°C for 3 min initial denaturation followed by 35 cycles of 94°C for 30 s, 58°C for 40 s, 72°C for 55 s, and an additional extension at 72°C for 10 min. PCR product sizes were verified on agarose gel then pooled by inoculum origin and quantified using a Nano-Drop1000. Twenty ng of pooled DNA from each inoculum was inserted into plasmids and cloned using a StrataClone PCR Cloning Kit according the manufacturer's protocol. To identify AM fungal sequences in each inoculum type, we picked 48 individual colonies per inoculum type and PCR amplified them using M13 forward and reverse primers. Product sizes were verified on a 1% agarose gel and run through a standard cycle-sequencing program using the M13-forward primer. Sequences were analyzed on an ABI 3730xl sequencer and then edited using Geneious Pro 5.0.4. We aligned these sequences using MAFFT as implemented in Geneious and grouped unknown sequences into 29 clusters based on 99% sequence similarity. Ninety-nine percent similarity is a conservative approximation of phylogenetic differences among AM fungal operational taxonomic units (OTUs);

however OTUs based on 97% and 98% similarity clustered sequences that matched different known species from Genbank data (data not shown). Sequence accumulation curves (rarefaction analysis) showed our sampling was saturated at 99% similarity (Supplementary material Appendix A4). Up to two sequences for each cluster were selected and aligned with 36 known SSU sequences (obtained from Genbank and trimmed to the AML1-AML2 primers) from across the AM fungal phylum. We then built a maximum likelihood (ML) phylogenetic tree with 100 bootstrap runs from this alignment in MEGA 4.0 (Kumar et al. 2008) to visualize the sequences within the AM fungal phylogeny. DNA sequences obtained in this study were deposited in GenBank under accession numbers JN252437–JN252479.

### Soil preparation

We established sterile common field soil in which to inoculate microbial communities and establish plants. Field soils from each stage were sterilized using gamma-irradiation to 32 kGy which has been shown to eliminate nearly all soil microbes with the least impact on edaphic conditions (McNamara et al. 2003). We combined soil cores from each of the random points (described earlier) across the successional stages (a total of 30 cores) to form a 'common' soil type. This common soil was then filtered through a 6 cm sieve to remove large organic material that could bias individual pots. In addition, we sterilized standard 'play sand' by autoclaving (212°C) for one hour. Soils were used to fill mini-tree pots (Stuewe and Sons) for each experimental unit. Each replicate pot consisted of a layer of 300 ml of play sand topped with 600 ml of sterile field soil which mimics field conditions for soil horizons on successional dunes (Lichter 1998b).

### Plant stratification and germination

To remove seed borne microbes, all seeds were surface sterilized using a 5% sodium hypochlorite solution for 30 s, then 70% ethanol for 30 s and finally rinsed thoroughly in sterile, de-ionized water. For all plant species, we tested a variety of stratification techniques using combinations of cold/warm phases and dark/light phases to induce germination in seeds. Most seeds were stratified and germinated in autoclaved "Sunshine" mix LA4. Seeds were placed slightly below the soil surface in cell-pack flats. Cold/dark stratification (4°C) was conducted in environmental chambers (Supplementary material Appendix A1).

### Experimental setup and growth

Seedlings were transplanted into pots within three days of germination. Plants were allowed to grow for two weeks and any seedlings that died were replaced. After two weeks, each plant species was inoculated with one of the six microbial additions: 1) sterile water control, 2) microbial wash only, 3) microbial wash + Early AM fungal inoculum, 4) wash + Intermediate AM fungal inoculum, 5) microbial wash + AM fungal inoculum from late succession dunes,

6) microbial wash + mixed AM fungal inoculum from all three stages. One ml of AM fungal inoculum was added directly to the root area using a pipette inserted slightly below the soil surface. One ml of microbial wash was subsequently added in the same way. Each plant by microbe treatment combination (48 in total) was replicated 10 times for a total of 480 experimental units. Drip irrigation was used to provide each pot with 8 ml of water three times per day. Pots were arranged in a randomized complete block design (48 replications block<sup>-1</sup>). However, each block was rotated every two weeks to minimize the effects of environmental variation within the greenhouse (Potvin 1993).

Soils were amended with 20 ml of a half strength Hoagland's solution (the full-strength solution contained (mol m<sup>-3</sup>): MgSO<sub>4</sub>, 2.0; Ca(NO<sub>3</sub>)<sub>2</sub>, 5.0; KNO<sub>3</sub>, 5.0; NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1.0, together with micronutrients and iron-EDTA), a low phosphorus fertilizer, at three and five months.

After six months plants were harvested. Aboveground biomass was dried at 55°C for 3 days and then weighed. Plant roots were gently shaken free of soil and washed on a 1mm sieve for up to 15 min to remove sand particles and then air dried. When wet root biomass was above 50 mg, we took a root sample for staining of arbuscular mycorrhizal structures. The remaining root material was oven dried at 37°C for three days and weighed. Soils for each experimental unit (pot) were homogenized and 100 mg was taken for quantification of soil fungal hyphal length.

## Analysis of fungal structures

Roots were stained with Chlorazol Black E (Brundrett et al. 1984), and percent colonization of AM fungi, was determined using the magnified intersect method (McGonigle et al. 1990). AM fungal hyphae were distinguished from other hyphae based on the presence of coenocytic hyphae. We randomly selected eighteen (2 cm long) root fragments from each pot and mounted them onto two glass slides. For each experimental unit we assessed the presence of arbuscules (the site of exchange between plant and fungus), vesicles (storage structures) and intra-radical hyphae at 150 intersections. We determined soil hyphal length by dissolving soil aggregates with sodium hexametaphosphate and staining filtered hyphae with Chlorazol Black E. Hyphae were visualized as above and intersections were converted to hyphal length (Hart and Reader 2002). Because *Pinus* species (*P. resinosa* and *P. strobus*) are known ecto-mycorrhizal species, data on arbuscules were reviewed and discarded because these observations could have been confounded with Hartig nets from ecto-mycorrhizal fungi (unpubl.).

## Statistical analysis

We used multivariate analysis of variance (MANOVA) to test for differences in total biomass, root biomass, shoot biomass and the ratio of root biomass to total biomass caused by microbial treatments among plant species (block was used as a random effect). None of the dependent variables differed among microbial treatments, but we conducted separate ANOVAs simply to report the data for each variable individually (Table 1). For fungal structures, we used MANOVA with the number of arbuscules, vesicles, intra radical hyphae, or soil hyphal length as dependent variables and plant species and AM fungal successional stage (and block) as independent variables. Several independent variables were significant therefore we conducted separate ANOVAs for each dependent variable and used Tukey post hoc tests to identify significant differences between individual treatments. For fungal analyses, control treatments (Control and Wash only) confirmed AM fungal colonization, but were removed from analyses (both had no arbuscules or vesicles and minimal levels of hyphae (internal and external), consistent with low level infection of non-AM fungi). For our separate ANOVA analysis with arbuscules, we also excluded *Pinus* species as indicated above. All analyses were conducted in R ver. 2.8.0 (R Core Development Team) and graphics were created using MEGA5 for the phylogenetic tree (Kumar et al. 2008) and SigmaPlot 9.0 (Systat Software) for all graphs.

## Results

### Fungal community composition of inocula

AM fungi in early successional inoculum were phylogenetically diverse, containing sequences from six genera across the phylum Glomeromycota. This diversity was replaced in intermediate and late successional inocula by sequences only from *Glomus* group B (Fig. 1). Of the 29 total OTUs at 99% sequence similarity, early succession inoculum contained 12 unique OTUs, intermediate succession contained three unique OTUs and late succession contained four unique OTUs. Intermediate and late successional inocula also shared a single AM fungal OTU that accounted for 60 of the 75 Glomeromycota sequences in those successional stages. The primers also amplified non-AM fungal sequences (*Mortierella* sp. and *Pinaceae*) from intermediate and late successional inocula that represented nine of the twenty-nine OTUs. This non-specific amplification may be due to a relatively small proportion

Table 1. ANOVA table of the effects of plant species and microbial inocula on each plant response variable. Significant values are in bold. Analyses included plant species to control for variation among plant species that a priori were likely to be different.

Plant response variable	Plant species			Microbial inoculum			Plant × AM inoculum		
	DF (factor, error)	F	p	DF	F	p	DF	F	p
Total biomass	7,397	159.05	<b>&lt;0.0001</b>	5,397	1.15	0.3339	35,397	0.78	0.81
Sqrt(shoot biomass)	7,397	202.56	<b>&lt;0.0001</b>	5,405	1.07	0.38	35,405	0.73	0.73
Root biomass	7,397	97.61	<b>&lt;0.0001</b>	5,397	0.77	0.57	35,397	0.59	0.97
Root: total biomass ratio	7,397	51.40	<b>&lt;0.0001</b>	5,396	0.59	0.70	35,396	0.68	0.92

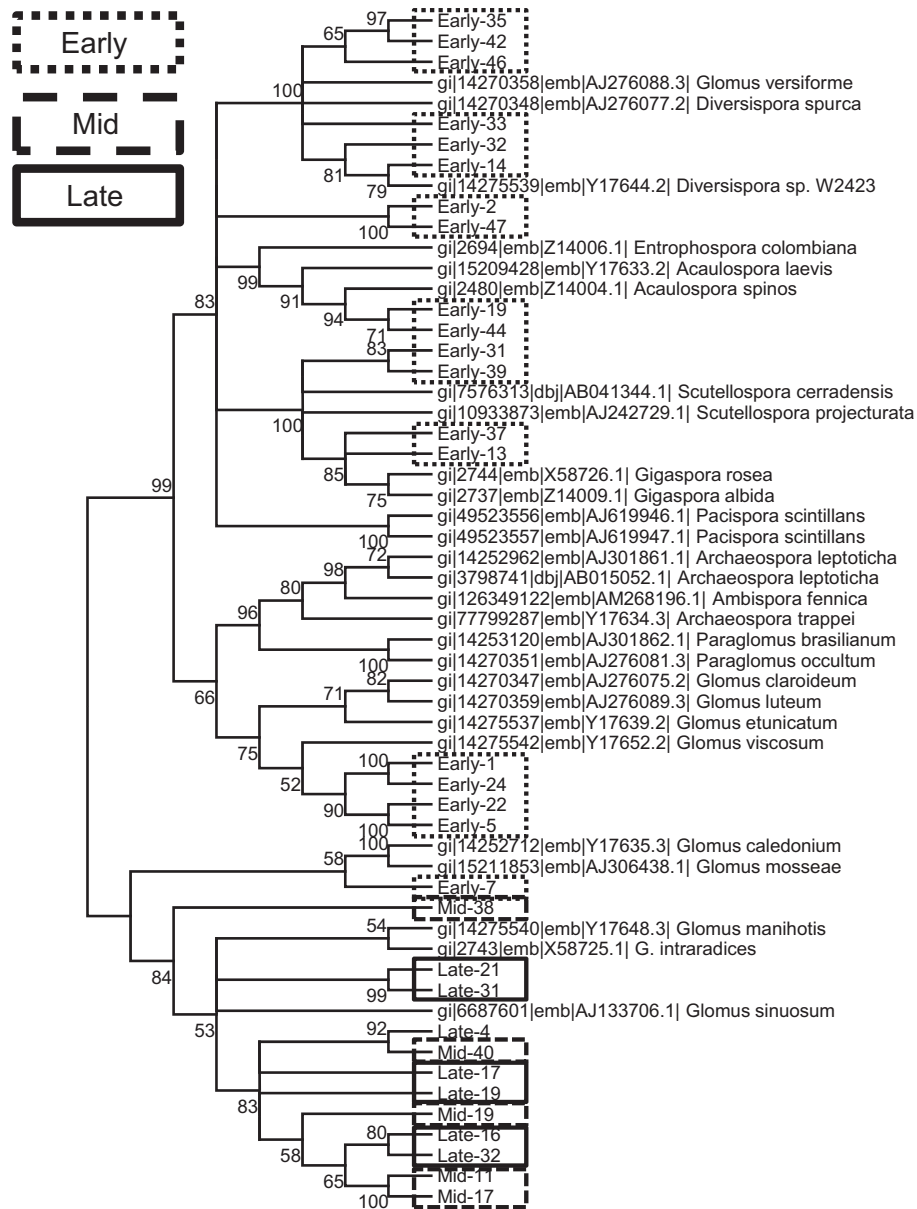


Figure 1. Maximum likelihood phylogenetic tree of 36 known AM fungi and unknown sequence clusters from Early, Mid or Late successional inocula. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Up to two individual sequences were taken as samples from each 99% similar cluster. For a complete list of sequences associated with each cluster see Supplementary material Appendix A3.

of AM fungal DNA in intermediate and late successional soils (Garner 2002).

### Plant responses to mycorrhiza

Though biomass differed among plant species, the source of AM fungal inocula did not affect any of the biomass metrics (approx  $F_{5,397} = 0.729$ ,  $p > 0.5$ ) including final shoot (Fig. 2) and final root biomass (Fig. 3). Data for each dependent variable ANOVA is reported in Table 1. Because tree seeds were obtained outside the study site, we also analysed data without these species, but this did not alter the significance of any of these tests (results not shown), therefore they were left in the reported analyses.

### Mycorrhizal fungal performance

Mycorrhizal structures within roots and in soil differed based on the interaction between fungal inocula origin and plant species ( $F_{21,262} = 1.66$ ,  $p < 0.0005$ ). Results for separate ANOVAs are presented in Table 2. The number of arbuscules differed only among AM fungal inocula ( $F_{3,192} = 73.55$ ,  $p < 0.0001$ , Fig. 4, Table 2) with more arbuscules formed by late successional AM fungi ( $p < 0.05$  all pair wise comparisons). Although vesicle frequency showed a marginally significant interaction between AM fungal inoculum and plant species ( $F_{15,262} = 1.57$ ,  $p = 0.055$ ), this effect was driven by differences between increased vesicles in *A. rubrum* plants when inoculated with late

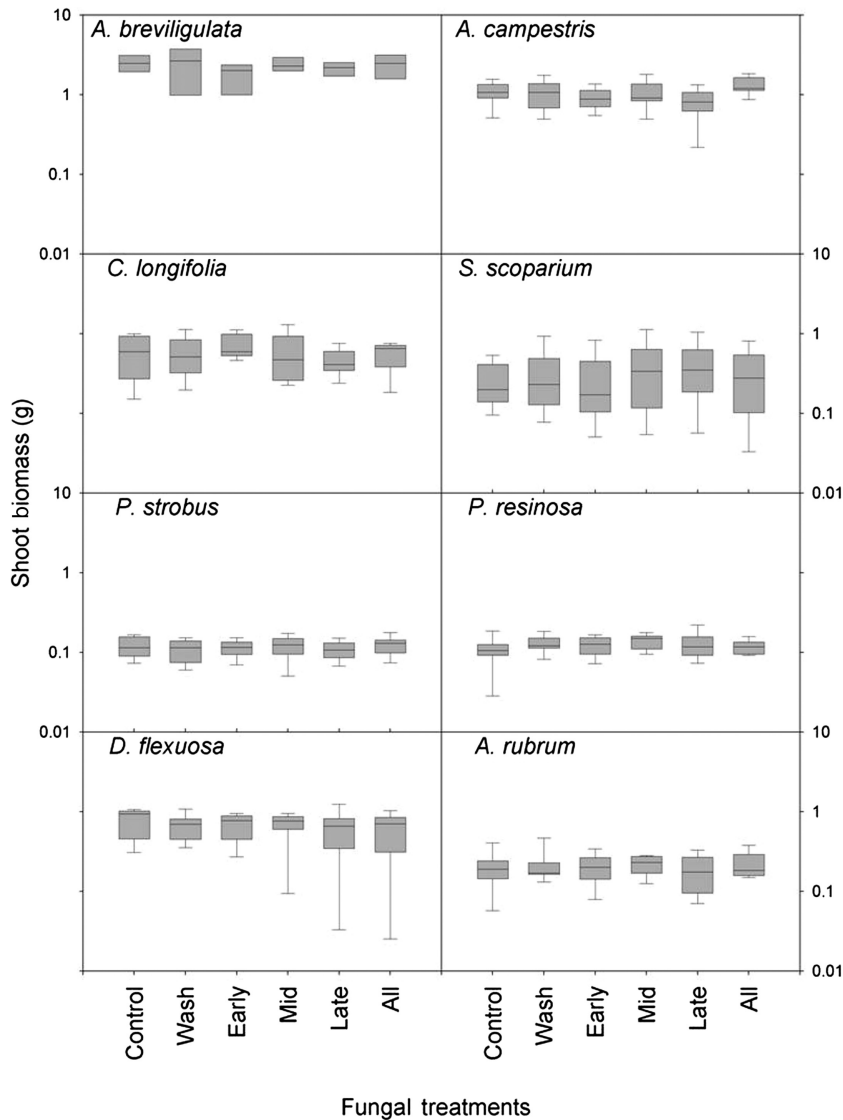


Figure 2. Shoot biomass of each plant species (log-scale) inoculated with each fungal treatment. Plant species are ordered from top to bottom in order of their dominance in succession. Fungal inocula correspond to AM fungal spores and hyphae isolated from specific successional stage as indicated in Methods. Boxes contain data from the 25th to 75th percentile, whiskers (error bars) contain 90% of the data for all treatments with at least nine surviving plants. Any outliers (beyond 5th or 95th percentile) are indicated as single points.

succession fungi. Intra-radical hyphae were also greatest in late successional inoculum ( $F_{3,262} = 2.86$ ,  $p < 0.05$ , Fig. 5) but did not differ among plant species ( $F_{5,191} = 0.74$ ,  $p > 0.5$ ). Although not quantified, the presence of septate hyphae within roots of control and wash treated plants indicated the presence of non-AM fungi, many of which are likely saprobes or pathogens (Klironomos 2002). As with arbuscules, the length of soil hyphae also differed among AM inocula ( $F_{3,264} = 26.88$ ,  $p < 0.0001$ , Table 1, Fig. 3) with late successional fungi having the longest hyphae per volume of soil ( $p < 0.05$  for all pair wise comparisons). The significant interaction between plant and AM fungal treatments on soil hyphal length ( $F_{3,264} = 1.73$ ,  $p < 0.05$ , Table 2) was caused by significantly fewer external hyphae in *Pinus* species which does not readily form arbuscular mycorrhizas.

## Discussion

AM fungal communities were phylogenetically different among dune successional stages and produced different quantities of mycorrhizal structures based on successional origin. AM fungi were more phylogenetically diverse in early successional soils, containing sequences from all major AM families in the Glomeromycota except the Paraglomeraceae. In contrast, intermediate and late successional soils contained only AM sequences within the group B clade of the Glomeraceae (Family Glomeraceae sensu Walker and Schüßler 2010). This high AM fungal diversity in early succession agrees with prior work in dune plantings (Koske and Gemma 1997) and contrasts with the lack of species changes in secondary, old-field succession (Johnson et al. 1991). These changes in AM fungal diversity also

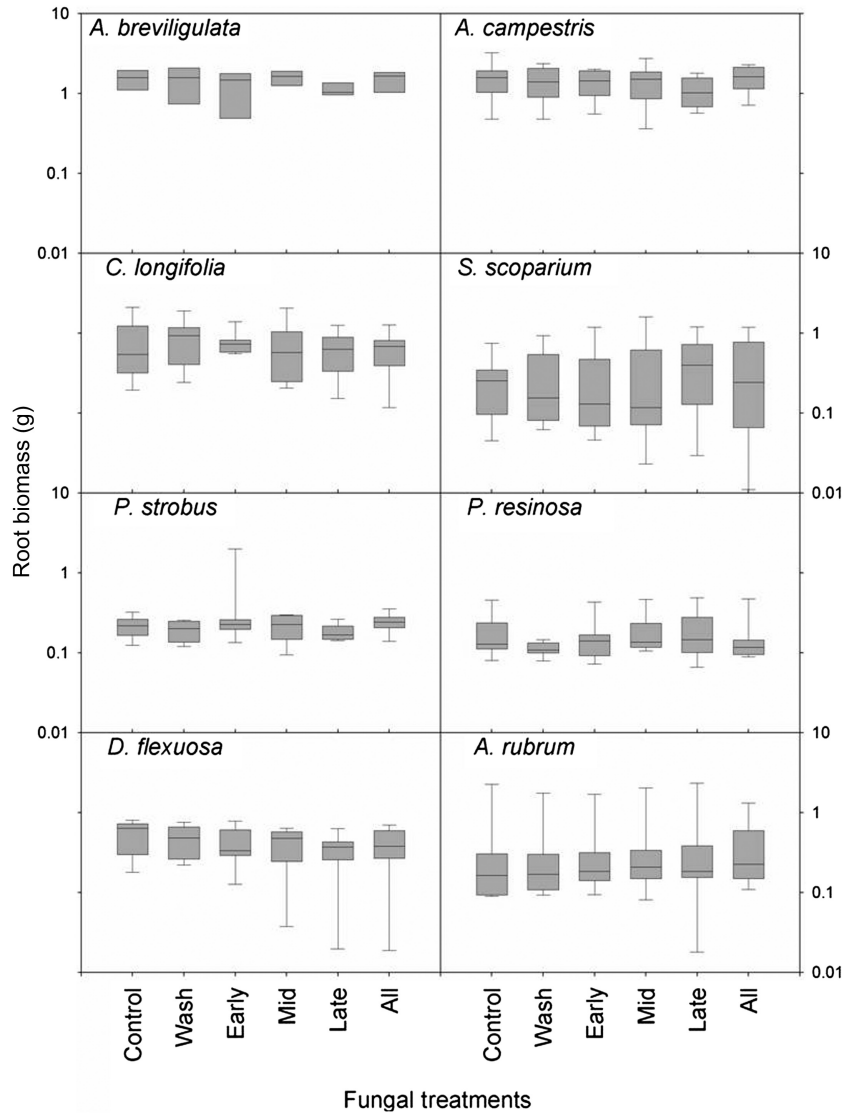


Figure 3. Root biomass of each plant species (log-scale) inoculated with each fungal treatment by plant species. Fungal inocula and figure symbols are as in Fig. 2.

contrast with plant diversity aboveground that is highest in intermediate succession (Lichter 1998b) where AM fungal diversity was low. It is unclear if this high diversity is a product of dispersal, niche based processes for multiple AM functions or based on asymmetries in specialization that occur with aboveground plant mutualists (Vázquez and Aizen 2004).

Even though intermediate and late succession AM fungi were phylogenetically similar, they were phenotypically

different. Late successional fungi produced more arbuscules and soil hyphae than intermediate successional fungi although both contained sequences from only a single clade and shared an abundant OTU. Although we cannot be sure all species colonized equally, the complete lack of overlap between early succession and the other stages means these differences are not from differential colonization alone. Phenotypic differences in mycorrhizal structures between AM fungi in inocula were also not necessarily consistent

Table 2. ANOVA table of plant species and AM inocula effects on mycorrhizal response variables. As indicated in Methods, Control and Wash treatments were removed from these analyses. Significant values are in bold.

Mycorrhizal response variable	Plant species			AM inoculum			Plant × AM inoculum		
	DF (factor, error)	F	p	DF	F	p	DF	F	p
Arbuscules	5,191	1.0497	0.3899	3,191	73.5506	<b>&lt;0.0001</b>	15,191	1.1718	0.2967
Vesicles	7,262	0.6341	0.727	3,262	0.0250	0.862	21,262	1.577	0.0547
% root colonization	7,262	1.0386	0.4044	3,262	2.8632	<b>0.0373</b>	21,262	1.496	0.0782
Soil hyphae	7,264	10.041	<b>&lt;0.0001</b>	3,264	22.321	<b>&lt;0.0001</b>	15,264	1.7323	<b>0.0263</b>

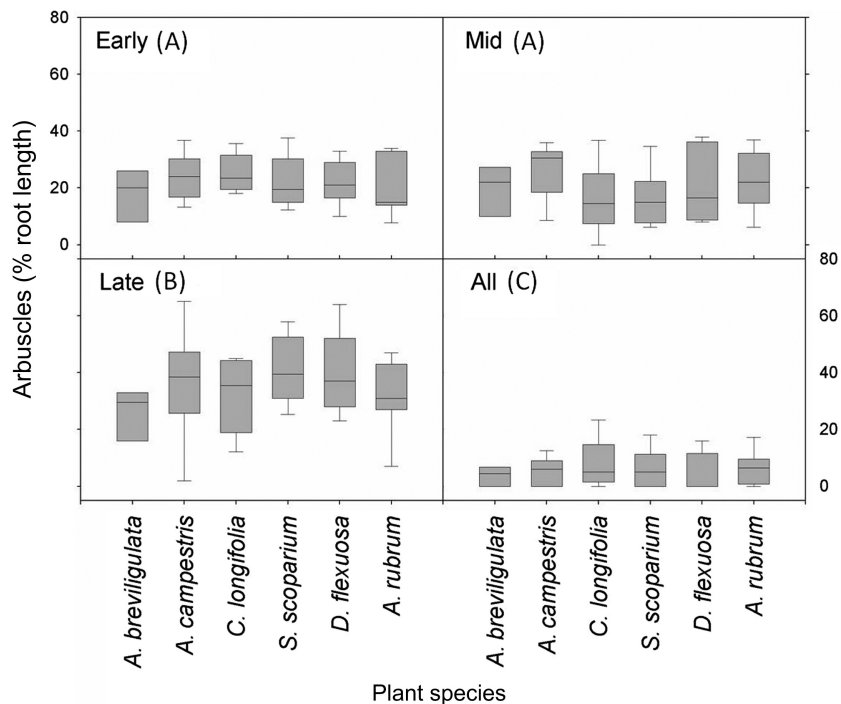


Figure 4. Percentage of plant root length with arbuscules for each AM fungal inoculum by plants species (Controls and Pinaceae species removed). Plants are ordered based on dominance in primary dune succession. Letters next to fungal inocula indicate significant differences between treatments ( $p < 0.0001$ ). Fungal inocula and symbols are as in Fig. 2 without the inclusion of control or wash treatments.

with previous descriptions of the same AM fungal phyla (Powell et al. 2009). AM fungi in early successional inoculum produced less soil hyphae than late successional inoculum even though the inoculum contained several sequences from the family Gigasporaceae which are normally associated with increased soil hyphae (Hart et al. 2001, Maherali and Klironomos 2007).

Although several mycorrhizal effects on plants are possible, differences in both mycorrhizal diversity and structures did not alter the growth of any plants from across primary sand dune successional stages relative to non-AM fungal treatments. We selected dominant plant species from across the successional series without regard to preference for particular mycorrhizal types (ecto- vs endomycorrhizae) or plant functional type (grasses vs trees), therefore we expected a priori that certain plant species (*Pinus alba* and *P. resinosa*) would not be responsive to AM fungi. However, several of the plant species which were previously responsive to AM fungi in shorter experiments (Brejda et al. 1993, Anderson et al. 1994, Little and Maun 1996), did not show significant mycorrhizal responses even though their roots were well colonized. The microbial wash produced septate hyphae, some of which are likely fungal pathogens, therefore mycorrhizal benefits from AM fungal pathogen protection could have occurred. In addition, the common sterilized field soil had low nutrient levels (initial N and P matched those of early and intermediate succession field soils; data not shown) and a minimal P fertilizer was added to all plants only when we first noticed visible nutrient stress (chlorosis). Given these low nutrients, it is unlikely that high P availability limited AM fungal benefit to plant growth (Johnson 1993, Collins and

Foster 2009). Finally, the mycorrhizal symbiosis costs plant photosynthate which could have suppressed plant growth. Based on these results, we suggest that the absence of direct AM fungal growth effects on plants reduces the likelihood that AM fungi influence sand dune plant succession through feedback effects.

We cannot rule out the possibility that AM fungal effects only occur through interactions with specific soil types or influence other processes that could affect plant succession, such as competition (Grime et al. 1987). Our experiment combined soils from multiple successional stages and individual plant species within each successional stage to analyze plant–mycorrhizal feedbacks among stages, but this approach also removed the possibility for three-way interactions between plants, soil types and fungi (Johnson et al. 2010) as well as plant specific fungal feedback (Jiang et al. 2010). The use of spores and hyphae was necessary to inoculate AM fungi only, but could have missed fungal species that are present only in roots or sporulate infrequently (Kowalchuk et al. 2002) and are important to succession. In addition, if mycorrhizal fungi do not provide an independent growth advantage for a single plant but enhance (or reduce) its ability to compete with its neighbours, they could still contribute to plant succession over time.

In conclusion, our experiment suggests that arbuscular mycorrhizal fungi also undergo primary succession, but feedback from these changes among successional stages may play a relatively minor role in primary plant succession on sand dunes. The contrast in diversity between AM fungal mutualists and plants may be a more widespread feature of soil microbes associating with plants. Changes in mycorrhizal diversity and structures along the successional



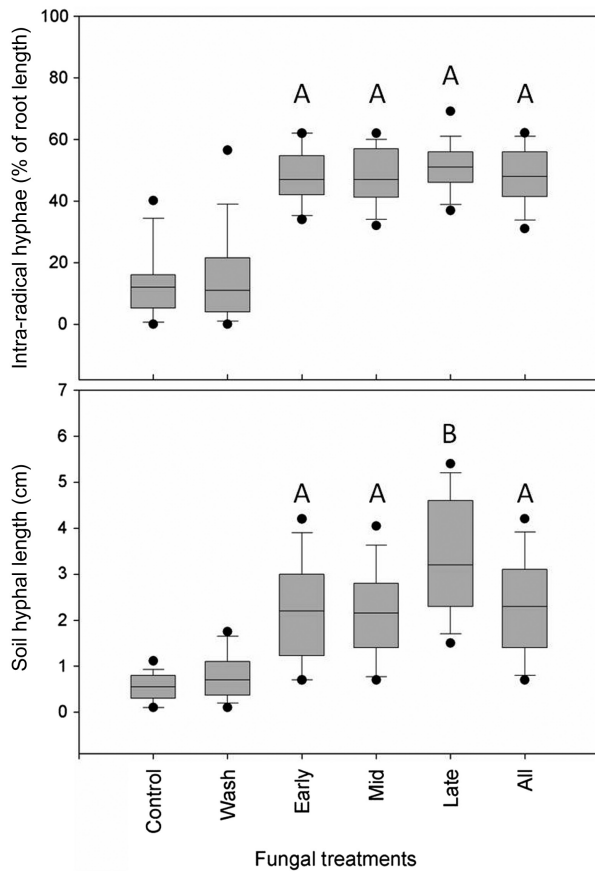


Figure 5. Percentage of root length colonized by fungal hyphae among all plant species for each fungal inocula. Control and Wash treatments are included for reference only. Letters indicate significant differences between fungal treatments ( $p < 0.0001$ ). Fungal inocula and figure symbols are as in Fig. 2.

sequence suggest that these mutualists could differentially alter successional processes through mechanisms not manifested in our greenhouse experiment. Other soil mutualists, such as nitrogen-fixing bacteria are present in early dune succession, affect primary succession and are also dependent on soil phosphorus that AM fungi could provide (Chapin et al. 1994, Uliassi and Ruess 2002, Dalton et al. 2004). Given the lack of direct effects, feedback from soil mutualisms during primary dune succession may be less important than feedback from soil pathogens and parasites (van der Putten et al. 1993). An important next step is to examine how adaptation of mycorrhizas to changing soil conditions can contribute to plant succession compared to plant driven changes in fungal community structure alone. Most theory thus far has focused on plant–soil feedbacks through reciprocal community changes only (Bever 1994, 2002, Kardol et al. 2006), but mycorrhizal effects in particular may occur through edaphic adaptation within habitats (Johnson et al. 2010). If late successional mycorrhizas are adapted to their local soil environment rather than plant hosts, soil changes during succession may more directly affect mycorrhizal function than changes in plant host identity.

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Supplementary material (available online as Appendix O20160 at <[www.oikosoffice.lu.se/appendix](http://www.oikosoffice.lu.se/appendix)>). Appendix A1–A4.