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

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Abstract

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 affects 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.

47

48 **Introduction**

49 Soil organisms can strongly influence plant succession, the changes in plant
50 communities over time, which is a foundational process for terrestrial ecosystem
51 development. Soil biota alter plant community structure by facilitating nutrient
52 mobilization and uptake (Vitousek et al. 1987, Smith and Read 2008), altering
53 competition between plants (Fitter 1977) pathogenesis (Olff et al. 2000, Klironomos
54 2002, Petermann et al. 2008), and through direct competition with plants (Diaz et al.
55 1993, Alberton et al. 2007). Therefore soil organisms can influence established
56 mechanisms of plant succession such as competition for nutrients (Clements 1916,
57 Connell and Slatyer 1977, Tilman 1985) and also represent a novel determinant of plant
58 succession (De Deyn et al. 2003, van der Putten et al. 2009).

59 Soil biotic effects on plant succession usually operate through feedbacks whose
60 outcome depends on the specific soil biota and mechanism of their effect. Feedback
61 effects occur through reciprocal changes in plant and soil biotic communities and are
62 generally negative when plants are grown in "home" soils (Kulmatiski et al. 2008). In
63 secondary succession of old fields, negative feedback from soil pathogens and parasites
64 limit the growth of early succession plants over those in later succession (De Deyn et al.
65 2003, Kardol et al. 2006, 2007). Positive soil feedback on late succession plant growth
66 was attributed to the accumulation of soil mututalists such as mycorrhizal fungi (Kardol
67 et al. 2006). Parallel work in primary succession on sand dunes also shows that soil
68 pathogens can limit early succession plants(van der Putten et al. 1993) but mutualisms
69 with arbuscular mycorrhizal (AM) fungi offset these effects (Little and Maun 1996). AM

70 fungi can also facilitate phosphorus uptake that may benefit plants in both late primary
71 and secondary succession when phosphorus is more limiting (Janos 1980, Vitousek and
72 Farrington 1997, Lichter 1998a). AM fungi therefore may either accelerate or retard
73 succession depending on the specific function they provide.

74 Particular phylogenetic lineages of AM fungi and fungal morphologies are more
75 effective at specific functions (Maherali and Klironomos 2007, Powell et al. 2009),
76 therefore specific fungal shifts driven by plant feedback could affect plant succession in
77 predictable ways. Plant hosts and fungal symbionts can preferentially allocate resources
78 to partners that provide a needed function resulting in positive feedbacks on plant and
79 fungal growth (Kiers et al. 2011). Therefore if pathogen protecting AM fungi form
80 symbioses with early succession plants, it should result in a positive feedback, stabilizing
81 plant-mycorrhizal interactions and retarding succession. However, the majority of plants
82 tested so far appear to cultivate less beneficial AM fungi over time resulting in negative
83 feedbacks (Bever 2002). Such negative feedbacks could accelerate succession by
84 favouring newly dispersed plant species over established species.

85 Here we examine how AM fungi differ among primary successional stages and
86 whether these fungal communities positively or negatively affect the growth of dominant
87 plant species from each successional stage in a reciprocal transplant experiment of early,
88 intermediate or late succession plants and fungi. We used molecular analysis to
89 characterize AM propagules from each successional stage before growing plants with
90 them. If AM fungi increase the growth of plant hosts from their own successional stage,
91 this indicates positive feedback that could slow succession. In contrast, if AM fungi
92 increase growth of plant hosts from other successional stages this would indicate negative

93 feedback that may accelerate plant succession. After plants were grown with AM fungi,
94 we examined whether AM structures and fungal growth of communities from each
95 successional stage was affected by plant hosts. If plant hosts influence mycorrhizal fungal
96 growth, then the number of fungal structures should differ among plant hosts. If soil
97 nutrients and edaphic conditions influence fungi, then AM fungal growth and structures
98 should reflect successional differences in the origin of each inoculum.

99 **Materials and Methods**

100 To determine interactions between AM fungi and plants during succession, we
101 collected soils from Wilderness State Park, Michigan, USA (45°43’ N, 84°56’ W), a
102 previously described sand dune successional series (Lichter 1998b) where 102 ridges have
103 formed at an average rate of approximately one dune every 32 years (Lichter 1995). Soils
104 were collected during June 2007. We selected 3 pairs of dunes that represented distinctly
105 different stages in both plant community composition and edaphic conditions including
106 soil pH and soil nutrients (Lichter 1998b). The youngest dunes were 10 and 35 years old
107 respectively, intermediate- aged dunes formed 235-295 years before present, and late
108 successional dunes formed 450 and 845 years before present (Lichter 1997). On each
109 dune we selected five random points along a 100m transect established parallel to the
110 shoreline at the apex of each dune. At each point, we collected ten soil cores using
111 autoclaved aluminum coring cans and carefully transferred them into 69 oz. Whirl-Pak
112 bags (Nasco, Modesto, California). Soils were refrigerated (4°C) within 24hrs,
113 transported back to the University of Guelph, Ontario, Canada and held at 4°C.

114 **Seed Collections and Plant Succession Assignment**

115 Within this dune succession, we chose to use plant species that were dominant in
116 each successional stage (but not in others) to increase the likelihood that any mycorrhizal
117 effects we observed could be applied broadly to plant succession. We collected seeds
118 from plant species across the series at Wilderness State Park over the summer of 2007.
119 Dominant plant species for each successional stage were chosen using percent cover data
120 along the successional series (Lichter 1998b) and personal observations (Supplemental
121 material Appendix 2). For early successional species we used *Ammophila breviligulata*,
122 *Artemisia campestris.* Both occur in the earliest dunes and are rarely present in dunes
123 older than 100 years. For mid succession, we selected *Calamovilfa longifolia,*
124 *Schizachyrium scoparium,* and *Pinus strobes*. *C. longifolia* replaces *A. brevigulata* as the
125 dominant dune grass in early succession (<100 ybp) but is still relatively abundant as *P.*
126 *strobus* colonizes and forms a closed canopy forest (~225 years after formation).
127 *Schizachyrium scoparium* becomes dominant at the last stages of open dunes, but is
128 absent after the canopy closes (~250 ybp). For late succession we used *Pinus resinosa,*
129 *Deschampsia flexuosa* and *Acer rubrum. P resinosa* replaces *P. strobes* as the dominant
130 canopy tree which, in turn is eventually replaced by *A. rubrum*. *D. flexuosa* is the only
131 major dominant understory grass in late succession. In sites older than ~ 835 years there
132 has been significant anthropogenic influence such as burning and logging to promote the
133 growth of particular harvestable species (Lichter 1998b). For this reason they were
134 excluded from our successional analysis.
135 Although these plant species dominate their respective successional stage, they
136 also have important differences in life-history characteristics. Within this
137 chronosequence, dominant grasses in early succession are replaced by tree species in

138 intermediate and late succession. These species also differ strongly in their known
139 mycorrhizal interactions, for example *Pinus* species associate with ecto-mycorrhizal
140 species rather than AM fungi used here. These differences in plant traits may make it
141 more likely to see differential effects of mycorrhizal inocula related to plant functional
142 types rather than only successional origin. For individual feedback responses, one can
143 compare plant species individually with mycorrhizal inocula from different successional
144 stages rather than among plant species. Due to seed predation in the field and low
145 germination success, we obtained seeds for dominant tree species (*Acer rubrum, Pinus*
146 *resinosa, Pinus strobus*) from the Ontario Ministry of Natural Resources Tree Seed
147 Facility which field collected seeds sources from a directly adjacent region in Canada.
148 However, because these seeds were not collected within this successional series (local
149 feedback) we conducted statistical analyses both with and without their inclusion.

150 **Inoculum Preparation**

151 To isolate AM fungal inoculum from each successional stage, we combined and
152 homogenized 2.4 kg of soil from dunes of similar age. Each combined soil contained an
153 equal amount of soil from five random points along each dune to pool spatial variation
154 within dunes of the same age. We then took 600g of soil from each of the combined soils
155 (1.8kg total) to establish a common mycorrhizal inoculum containing all possible AM
156 fungi. We used repeated sucrose-centrifugation of soils (200g/isolation) to collect spores
157 and hyphae (Brundrett et al. 1994) for fungal inocula. Spores and hyphae were rinsed
158 thoroughly with autoclaved, de-ionized water, sonicated for one minute to remove surface
159 debris, rinsed again and then re-hydrated in 100ml of autoclaved, de-ionized water (final
160 concentration 18g soil/ml inoculum). To control for differences in microbial

161 contaminants introduced with AM inoculum, we also collected a microbial filtrate by
162 passing the initial spore filtrates through a 25μm sieve. Microbial filtrates from all dunes
163 were combined to represent a common microbial wash added as a control (Koide and Li
164 1989) to all AM inocula. AM fungal inoculum and microbial wash was maintained at 4°C
165 for two weeks prior to plant inoculation. Final inocula were water only (hereafter
166 'Control'), microbial wash alone (hereafter 'Wash'), microbial wash + AM fungal
167 inoculum from early successional dunes (hereafter 'Early'), microbial wash + AM fungal
168 inoculum from intermediate age dunes (hereafter 'Intermediate"), microbial wash + AM
169 fungal inoculum from late successional dunes (hereafter 'Late'), and microbial wash +
170 combined AM fungal inocula from all stages (hereafter 'All').

171 **Molecular Analysis of Fungal Inocula**

172 We used DNA cloning and sequencing to characterize and compare the AM
173 fungal inoculum of each successional stage that we used in the experiment. 10 ml of
174 inoculum from each successional stage was used for each DNA extraction and analysis.
175 Each sample was spun at 2,500 x g for 10 minutes and the supernatant removed. We
176 extracted DNA from inocula (spores and hyphae) using a PowerMax Soil kit (MoBio,
177 Carlsbad, CA) according to the manufacturer's instructions with an initial step where
178 inocula were ground under liquid nitrogen. Four replicate PCR amplifications were run
179 for each inoculum extract using the Glomeromycota specific primers, AML1 and AML2
180 (Lee et al. 2008) and the following PCR reaction mix: 20μl total reaction volume; Final
181 concentrations: 1X PCR buffer; 1.5 mM $MgCl_2$; 0.2mM dNTP's; 0.5 μM of each primer
182 and 0.75U of Platinum *Taq* Polymerase (Invitrogen). Thermocyling parameters were
183 slightly altered from those published in Lee et al. (2008): 94°C for 3 min initial

184 denaturation followed by 35 cycles of 94ºC for 30s, 58ºC for 40s, 72ºC for 55s, and an
185 additional extension at 72ºC for 10min. PCR product sizes were verified on agarose gel
186 then pooled by inoculum origin and quantified using a Nano-Drop1000 (Thermo-
187 Scientific, Wilmington, DE). 20ng of pooled DNA from each inoculum was inserted into
188 plasmids and cloned using a StrataClone PCR Cloning Kit (Agilent Technologies, Santa
189 Clara, CA) according the manufacturer's protocol. To identify AM fungal sequences in
190 each inoculum type, we picked 48 individual colonies per inoculum type and PCR
191 amplified them using M13 forward and reverse primers. Product sizes were verified on a
192 1% agarose gel and run through a standard cycle-sequencing program using the M13-
193 forward primer. Sequences were analyzed on an ABI 3730xl sequencer and then edited
194 using Geneious Pro 5.0.4 (Biomatters Ltd. 2010). We aligned these sequences using
195 MAFFT as implemented in Geneious and grouped unknown sequences into 29 clusters
196 based on 99% sequence similarity. 99% similarity is a conservative approximation of
197 phylogenetic differences among AM fungal operational taxonomic units (OTUs);
198 however OTUs based on 97% and 98% similarity clustered sequences that matched
199 different known species from Genbank data (data not shown). Sequence accumulation
200 curves (rarefaction analysis) showed our sampling was saturated at 99% similarity
201 (Supplemental material Appendix 4). Up to two sequences for each cluster were selected
202 and aligned with 36 known SSU sequences (obtained from Genbank and trimmed to the
203 AML1-AML2 primers) from across the AM fungal phylum. We then built a maximum
204 likelihood (ML) phylogenetic tree with 100 bootstrap runs from this alignment in MEGA
205 4.0 (Kumar et al. 2008) to visualize the sequences within the AM fungal phylogeny.

206 DNA sequences obtained in this study were deposited in GenBank under accession
207 numbers JN252437-JN252479.

208 **Soil Preparation**

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

221 **Plant Stratification and Germination**

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

229 chambers (Supplemental material Appendix 1). Five of the thirteen species collected in
230 the field germinated in sufficient quantity for inclusion in the experiment. The tree seeds
231 collected from the adjacent sites all germinated in sufficient quantities.

232 **Experimental Setup and Growth**

233 Seedlings were transplanted into pots within three days of germination. Plants
234 were allowed to grow for two weeks and any seedlings that died were replaced. After two
235 weeks, each plant species was inoculated with one of the six microbial additions: 1)
236 sterile water control, 2) microbial wash only, 3) microbial wash + Early AM fungal
237 inoculum, 4) wash + Intermediate AM fungal inoculum, 5) microbial wash + AM fungal
238 inoculum from late succession dunes, 6) microbial wash + mixed AM fungal inoculum
239 from all three stages. One ml of AM fungal inoculum was added directly to the root area
240 using a pipette inserted slightly below the soil surface. One ml of microbial wash was
241 subsequently added in the same way. Each plant by microbe treatment combination (48 in
242 total) was replicated 10 times for a total of 480 experimental units. Drip irrigation was
243 used to provide each pot with 8ml of water three times per day. Pots were arranged in a
244 randomized complete block design (48 reps/ block). However, each block was rotated
245 every two weeks to minimize the effects of environmental variation within the
246 greenhouse (Potvin 1993).

247 Soils were amended with 20ml of a half strength Hoagland's solution (the full-
248 strength solution contained (mol m^{-3}): $MgSO_4$, 2.0; $Ca(NO_3)_2$, 5.0; KNO_3, 5.0;
249 $NH_4H_2PO_4$, 1.0, together with micronutrients and iron-EDTA), a low phosphorus
250 fertilizer, at three and five months.

251 After six months plants were harvested. Aboveground biomass was dried at 55°C
252 for 3 days and then weighed. Plant roots were gently shaken free of soil and washed on a
253 1mm sieve for up to 15 min to remove sand particles and then air dried. When wet root
254 biomass was above 50 mg, we took a root sample for staining of arbuscular mycorrhizal
255 structures. The remaining root material was oven dried at 37°C for three days and
256 weighed. Soils for each experimental unit (pot) were homogenized and 100mg was taken
257 for quantification of soil fungal hyphal length.

258 **Analysis of Fungal Structures**

259 Roots were stained with Chlorazol Black E (Brundrett et al., 1984), and percent
260 colonization of AM fungi, was determined using the magnified intersect method
261 (McGonigle et al., 1990). AM fungal hyphae were distinguished from other hyphae based
262 on the presence of coenocytic hyphae. We randomly selected eighteen (2cm long) root
263 fragments from each pot and mounted them onto two glass slides. For each experimental
264 unit we assessed the presence of arbuscules (the site of exchange between plant and
265 fungus), vesicles (storage structures) and intra-radical hyphae at 150 intersections. We
266 determined soil hyphal length by dissolving soil aggregates with sodium
267 hexametaphophate and staining filtered hyphae with Chlorazol Black E. Hyphae were
268 visualized as above and intersections were converted to hyphal length (Hart and Reader
269 2002). Because *Pinus* species (*P. resinosa* & *P. strobus*) are known ecto-mycorrhizal
270 species, data on arbuscules were reviewed and discarded because these observations
271 could have been confounded with Hartig nets from ecto-mycorrhizal fungi (personal
272 observation).

273 **Statistical Analysis**

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

292 **<u>Results</u>**

293 *Fungal community composition of inocula*

294 AM fungi in early successional inoculum were phylogentically diverse,
295 containing sequences from six genera across the phylum Glomeromycota. This diversity
296 was replaced in intermediate and late successional inocula by sequences only from

297 *Glomus* Group B (Figure 1). Of the twenty-nine total OTUs at 99% sequence similarity,
298 early succession inoculum contained twelve unique OTUs, intermediate succession
299 contained three unique OTUs and late succession contained four unique OTUs.
300 Intermediate and late successional inocula also shared a single AM fungal OTU that
301 accounted for 60 of the 75 Glomeromycota sequences in those successional stages. The
302 primers also amplified non-AM fungal sequences (*Mortierella sp.* and *Pinaceae*) from
303 intermediate and late successional inocula that represented nine of the twenty-nine OTUs.
304 This non-specific amplification may be due to a relatively small proportion of AM fungal
305 DNA in intermediate and late successional soils (Garner 2002).

306 *Plant Responses to Mycorrhiza*

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

313 *Mycorrhizal Fungal Performance*

314 Mycorrrhizal structures within roots and in soil differed based on the interaction
315 between fungal inocula origin and plant species ($F_{21,262}=1.66$, $p<0.0005$). Results for
316 separate ANOVAs are presented in Table 2. The number of arbuscules differed only
317 among AM fungal inocula ($F_{3,192}=73.55$, $p<0.0001$, Figure 4, Table 2) with more
318 arbuscules formed by late successional AM fungi ($p<0.05$ all pair wise comparisons).
319 Although vesicle frequency showed a marginally significant interaction between AM

320 fungal inoculum and plant species ($F_{15,262}$=1.57, p=0.055), this effect was driven by
321 differences between increased vesicles in *A. rubrum* plants when inoculated with late
322 succession fungi. Intra-radical hyphae were also greatest in late successional inoculum
323 ($F_{3,262}$=2.86, p<0.05, Figure 5) but did not differ among plant species ($F_{5,191}$=0.74, p>0.5).
324 Although not quantified, the presence of septate hyphae within roots of control and wash
325 treated plants indicated the presence of non-AM fungi, many of which are likely saprobes
326 or pathogens (Klironomos 2002). As with arbuscules, the length of soil hyphae also
327 differed among AM inocula ($F_{3,264}$=26.88, p<0.0001, Table 1, Figure 3) with late
328 successional fungi having the longest hyphae per volume of soil (p<0.05 for all pair wise
329 comparisons). The significant interaction between plant and AM fungal treatments on soil
330 hyphal length ($F_{3,264}$=1.73, p<0.05, Table 2) was caused by significantly fewer external
331 hyphae in *Pinus* species which does not readily form arbuscular mycorrhizas.

332 **Discussion**

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

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

347 Even though intermediate and late succession AM fungi were phylogenetically
348 similar, they were phenotypically different. Late successional fungi produced more
349 arbuscules and soil hyphae than intermediate successional fungi although both contained
350 sequences from only a single clade and shared an abundant OTU. Although we cannot be
351 sure all species colonized equally, the complete lack of overlap between early succession
352 and the other stages means these differences are not from differential colonization alone.
353 Phenotypic differences in mycorrhizal structures between AM fungi in inocula were also
354 not necessarily consistent with previous descriptions of the same AM fungal phyla
355 (Powell et al., 2009). AM fungi in early successional inoculum produced less soil hyphae
356 than late successional inoculum even though the inoculum contained several sequences
357 from the family Gigasporaceae which are normally associated with increased soil hyphae
358 (Hart et al. 2001, Maherali and Klironomos 2007).

359 Although several mycorrhizal effects on plants are possible, differences in both
360 mycorrhizal diversity and structures did not alter the growth of any plants from across
361 primary sand dune successional stages relative to non-AM fungal treatments. We selected
362 dominant plant species from across the successional series without regard to preference
363 for particular mycorrhizal types (ecto- vs. endomycorrhizae) or plant functional type
364 (grasses vs. trees), therefore we expected *a priori* that certain plant species (*Pinus alba*
365 and *Pinus resinosa*) would not be responsive to AM fungi. However, several of the plant

366 species which were previously responsive to AM fungi in shorter experiments (Brejda et
367 al. 1993, Anderson et al. 1994, Little and Maun 1996), did not show significant
368 mycorrhizal responses even though their roots were well colonized. The microbial wash
369 produced septate hyphae, some of which are likely fungal pathogens, therefore
370 mycorrhizal benefits from AM fungal pathogen protection could have occurred. In
371 addition, the common sterilized field soil had low nutrient levels (initial N and P matched
372 those of early and intermediate succession field soils; data not shown) and a minimal P
373 fertilizer was added to all plants only when we first noticed visible nutrient stress
374 (chlorosis). Given these low nutrients, it is unlikely that high P availability limited AM
375 fungal benefit to plant growth (Johnson 1993, Collins and Foster 2009). Finally, the
376 mycorrhizal symbiosis costs plant photosynthate which could have suppressed plant
377 growth. Based on these results, we suggest that the absence of direct AM fungal growth
378 effects on plants reduces the likelihood that AM fungi influence sand dune plant
379 succession through feedback effects.

380 We cannot rule out the possibility that AM fungal effects only occur through
381 interactions with specific soil types or influence other processes that could affect plant
382 succession, such as competition (Grime et al. 1987). Our experiment combined soils from
383 multiple successional stages and individual plant species within each successional stage
384 to analyze plant-mycorrhizal feedbacks among stages, but this approach also removed the
385 possibility for three-way interactions between plants, soil types and fungi (Johnson et al.
386 2010) as well as plant specific fungal feedback (Jiang et al. 2010). The use of spores and
387 hyphae was necessary to inoculate AM fungi only, but could have missed fungal species
388 that are present only in roots or sporulate infrequently (Kowalchuk et al. 2002) and are

389 important to succession. In addition, if mycorrhizal fungi do not provide an independent
390 growth advantage for a single plant but enhance (or reduce) its ability to compete with its
391 neighbours, they could still contribute to plant succession over time.

392 In conclusion, our experiment suggests that arbuscular mycorrhizal fungi also
393 undergo primary succession, but feedback from these changes among successional stages
394 may play a relatively minor role in primary plant succession on sand dunes. The contrast
395 in diversity between AM fungal mutalists and plants may be a more widespread feature
396 of soil microbes associating with plants. Changes in mycorrhizal diversity and structures
397 along the successional sequence suggest that these mutualists could differentially alter
398 successional processes through mechanisms not manifested in our greenhouse
399 experiment. Other soil mutualisms, such as nitrogen-fixing bacteria are present in early
400 dune succession and affect other primary successions are also dependent on soil
401 phosphorus that AM fungi could provide (Chapin et al. 1994, Uliassi and Ruess 2002,
402 Dalton et al. 2004). Given the lack of direct effects, feedback from soil mutualisms
403 during primary dune succession may be less important than feedback from soil pathogens
404 and parasites (van der Putten et al. 1993). An important next step is to examine how
405 adaptation of mycorrhizas to changing soil conditions can contribute to plant succession
406 compared to plant driven changes in fungal community structure alone. Most theory thus
407 far has focused on plant-soil feedbacks through reciprocal community changes only
408 (Bever 1994, 2002, Kardol et al. 2006), but mycorrhizal effects in particular may occur
409 through edaphic adaptation within habitats (Johnson et al. 2010). If late successional
410 mycorrhizas are adapted to their local soil environment rather than plant hosts, soil

411 changes during succession may more directly affect mycorrhizal function than changes in
412 plant host identity.
413

414 **Acknowledgements**
415 The authors are grateful for funding from the Natural Sciences and Engineering
416 Research Council of Canada to JNK and HM as well as a David H. Smith Conservation
417 Fellowship from the Cedar Tree Foundation to BAS. We would like to thank the
418 comments of two anonymous reviewers for improving this manuscript considerably. We
419 also thank Kevin Courtney and several other undergraduate researchers for their help in
420 collecting soils and maintaining the experiments.
421

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538

	Plant species				Microbial Inoculum			plant*AM inoculum	
Plant Response Variable	df (factor, error)	F	p	df	F	p	df	F	p
Total Biomass	7, 397	159.05	**<.0001**	5, 397	1.15	0.3339	35, 397	0.78	0.81
Sqrt(Shoot Biomass)	7, 397	202.56	**<.0001**	5, 405	1.07	0.38	35, 405	0.73	0.73
Root Biomass	7, 397	97.61	**<.0001**	5, 397	0.77	0.57	35, 397	0.59	0.97
Root: Total Biomass Ratio	7, 397	51.40	**<.0001**	5, 396	0.59	0.70	35, 396	0.68	0.92

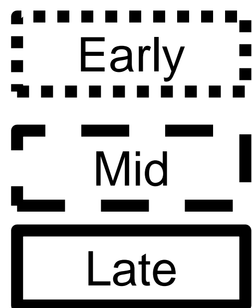
Table 1: ANOVA table of the Effects of Plant Species and Microbial Inocula on Each Plant Response Variable. Significant values are in bold. Analyses included plant species to control for variation among plant species that *a priori* were likely to be different.

Mycorrhizal Response Variable	Plant species df (factor, error)	F	p	AM inoculum df	F	p	plant*AM inoculum df	F	p
Arbuscules	5,191	1.0497	0.3899	3,191	73.5506	**<.0001**	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	**0.0373**	21,262	1.496	0.0782
Soil Hyphae	7,264	10.041	**<.0001**	3,264	22.321	**<.0001**	15,264	1.7323	**0.0263**

548

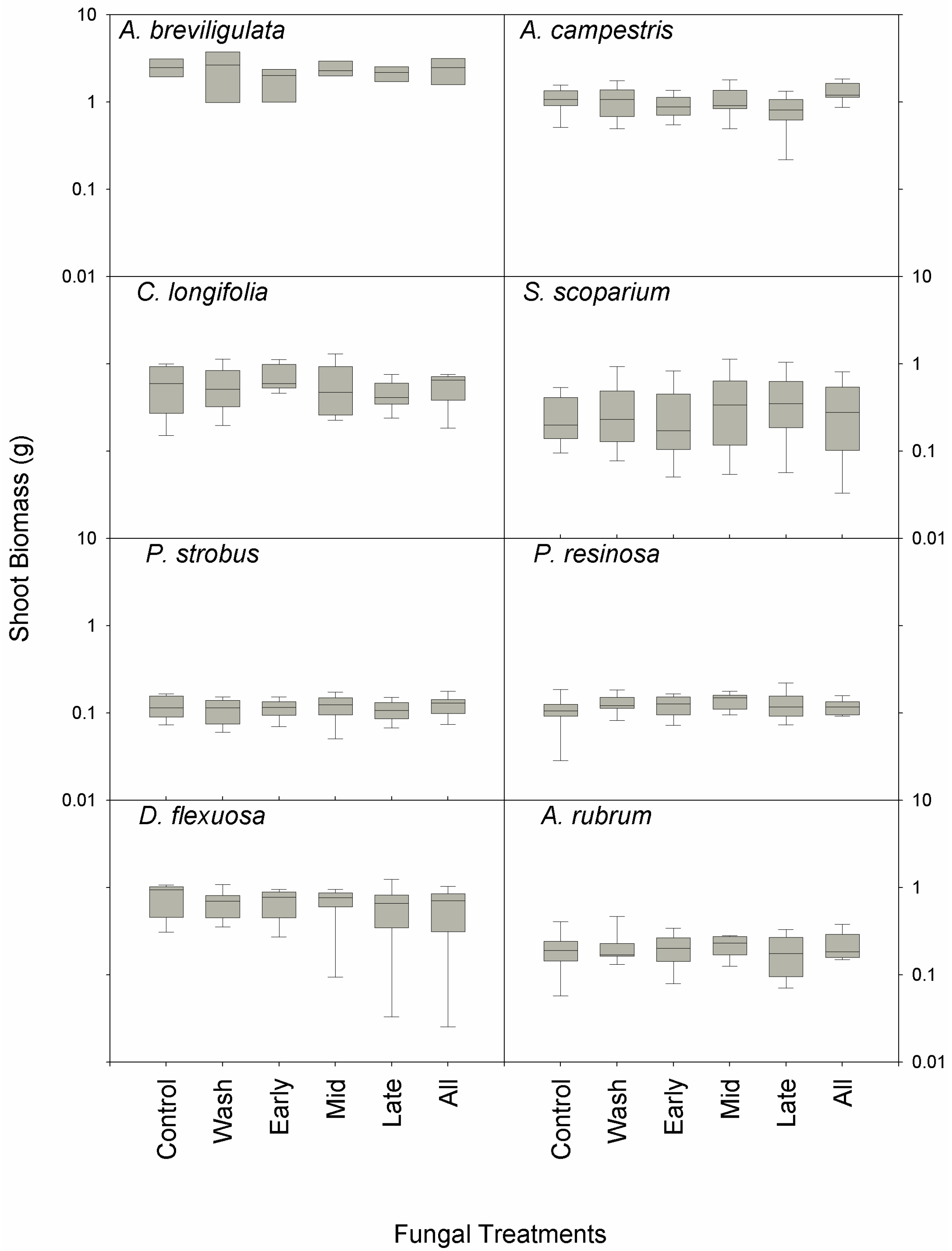
549 Table 2: ANOVA table of plant species and AM inocula effects on mycorrhizal response variables. As indicated in Methods, Control

550 and Wash treatments were removed from these analyses. Significant values are in bold.



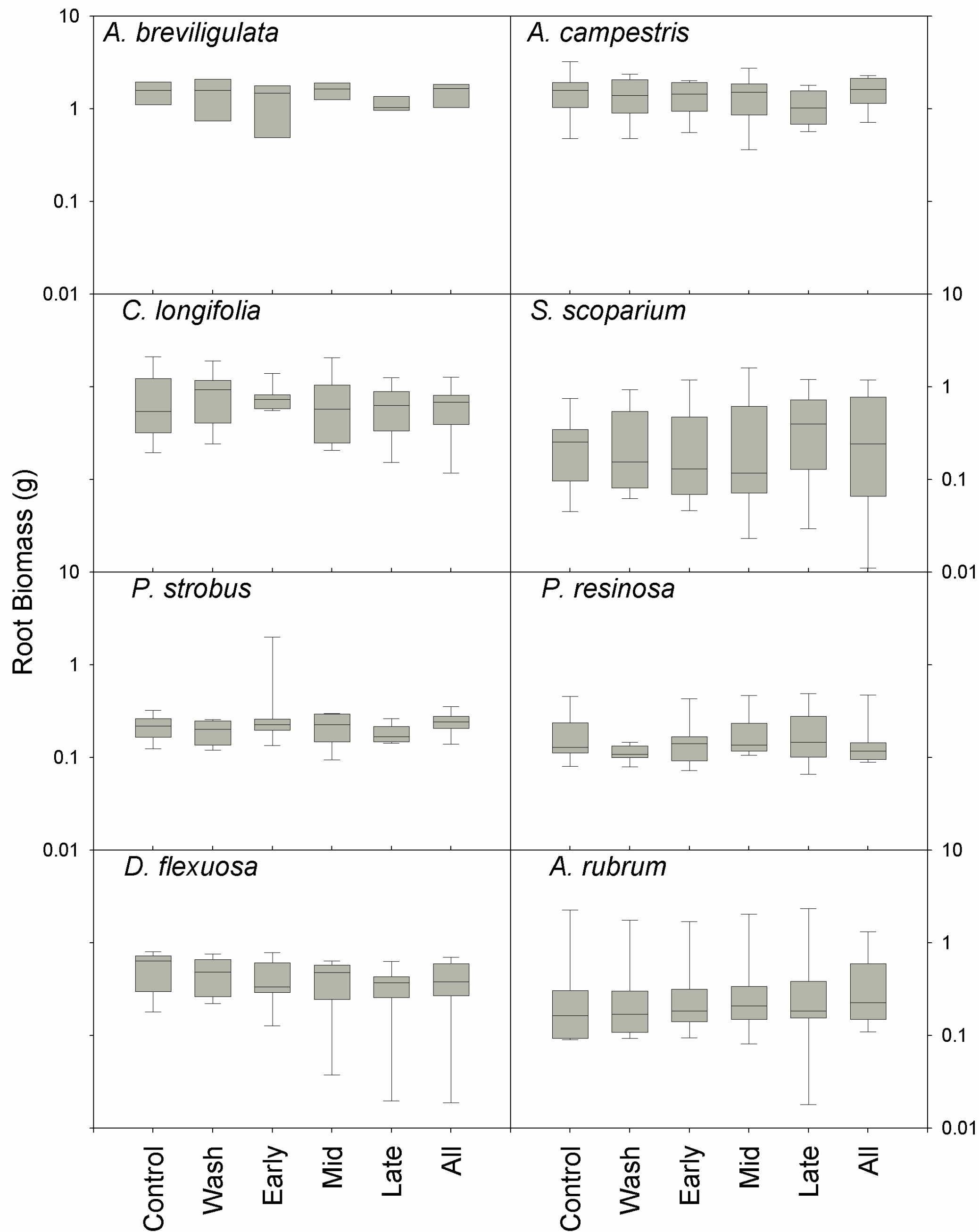
Early-35
Early-42
Early-46
gi|14270358|emb|AJ276088.3| Glomus versiforme
gi|14270348|emb|AJ276077.2| Diversispora spurca
Early-33
Early-32
Early-14
gi|14275539|emb|Y17644.2| Diversispora sp. W2423
Early-2
Early-47
gi|2694|emb|Z14006.1| Entrophospora colombiana
gi|15209428|emb|Y17633.2| Acaulospora laevis
gi|2480|emb|Z14004.1| Acaulospora spinos
Early-19
Early-44
Early-31
Early-39
gi|7576313|dbj|AB041344.1| Scutellospora cerradensis
gi|10933873|emb|AJ242729.1| Scutellospora projecturata
Early-37
Early-13
gi|2744|emb|X58726.1| Gigaspora rosea
gi|2737|emb|Z14009.1| Gigaspora albida
gi|49523556|emb|AJ619946.1| Pacispora scintillans
gi|49523557|emb|AJ619947.1| Pacispora scintillans
gi|14252962|emb|AJ301861.1| Archaeospora leptoticha
gi|3798741|dbj|AB015052.1| Archaeospora leptoticha
gi|126349122|emb|AM268196.1| Ambispora fennica
gi|77799287|emb|Y17634.3| Archaeospora trappei
gi|14253120|emb|AJ301862.1| Paraglomus brasilianum
gi|14270351|emb|AJ276081.3| Paraglomus occultum
gi|14270347|emb|AJ276075.2| Glomus claroideum
gi|14270359|emb|AJ276089.3| Glomus luteum
gi|14275537|emb|Y17639.2| Glomus etunicatum
gi|14275542|emb|Y17652.2| Glomus viscosum
Early-1
Early-24
Early-22
Early-5
gi|14252712|emb|Y17635.3| Glomus caledonium
gi|15211853|emb|AJ306438.1| Glomus mosseae
Early-7
Mid-38
gi|14275540|emb|Y17648.3| Glomus manihotis
gi|2743|emb|X58725.1| G.intraradices
Late-21
Late-31
gi|6687601|emb|AJ133706.1| Glomus sinuosum
Late-4
Mid-40
Late-17
Late-19
Mid-19
Late-16
Late-32
Mid-11
Mid-17

551 Figure 1: Maximum likelihood phylogenetic tree of 36 known AM fungi and unknown
552 sequence clusters from Early, Mid or Late successional inocula. Branches corresponding
553 to partitions reproduced in less than 50% bootstrap replicates are collapsed. The
554 percentage of replicate trees in which the associated taxa clustered together in the
555 bootstrap test (100 replicates) are shown next to the branches. Up to two individual
556 sequences were taken as samples from each 99% similar cluster. For a complete list of
557 sequences associated with each cluster see Supplemental material Appendix 3.



558

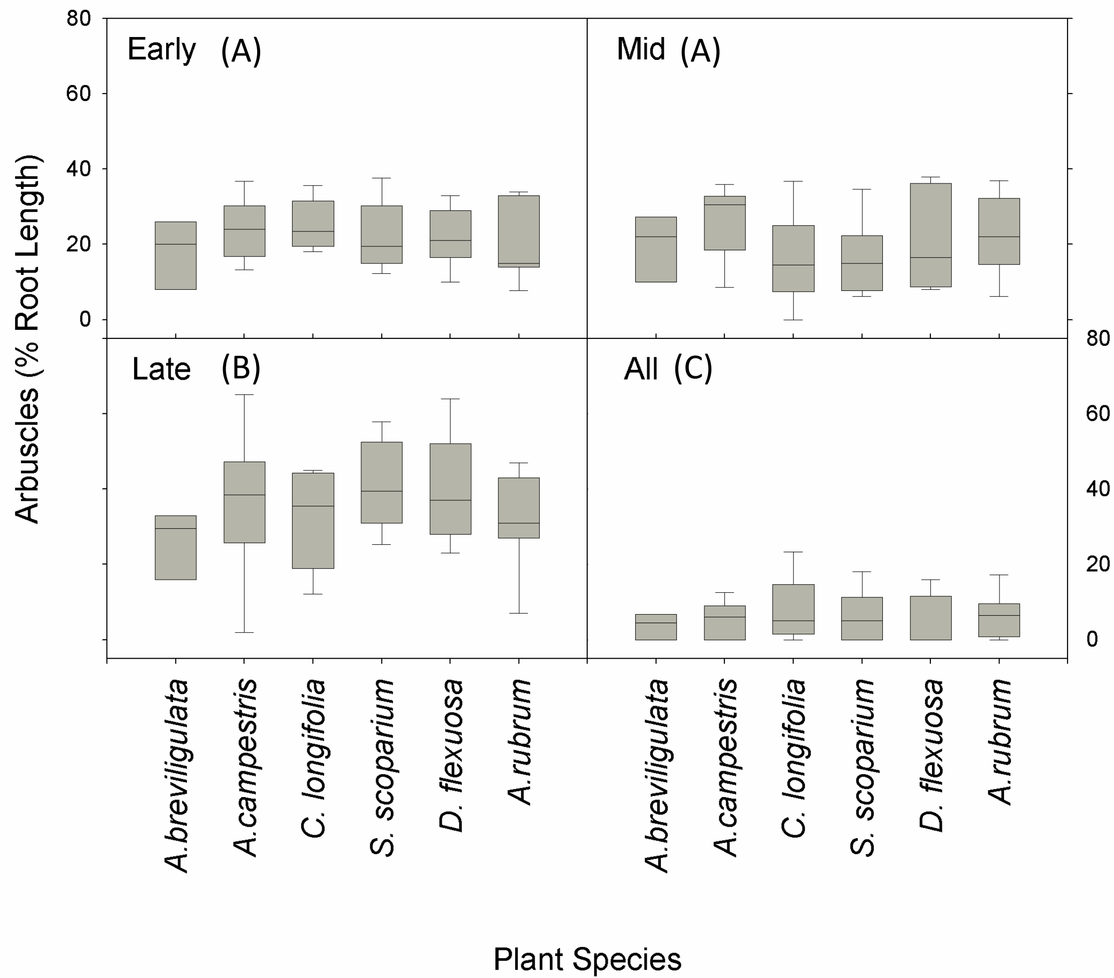
559 Figure 2: Shoot biomass of each plant species (log-scale) inoculated with each fungal
560 treatment. Plant species are ordered from top to bottom in order of their dominance in
561 succession. Fungal inocula correspond to AM fungal spores and hyphae isolated from
562 specific successional stage as indicated in Methods. Boxes contain data from the 25th to
563 75th percentile, whiskers (error bars) contain 90% of the data for all treatments with at
564 least nine surviving plants. Any outliers (beyond 5th or 95th percentile) are indicated as
565 single points.



566

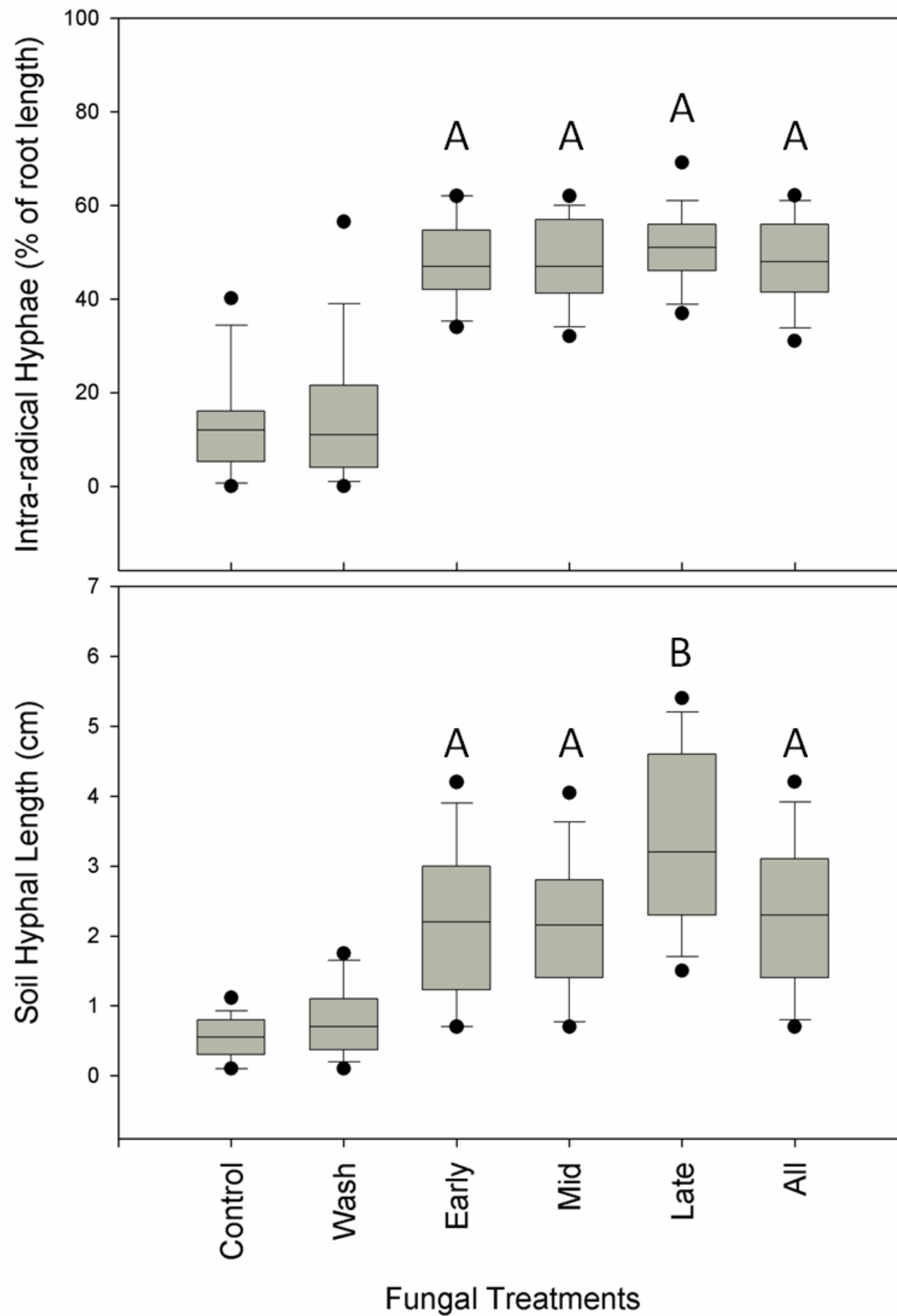
567 Figure 3: Root biomass of each plant species (log-scale) inoculated with each fungal

568 treatment by plant species. Fungal inocula and figure symbols are as in Figure 2.



569

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



575

576 Figure 5: Percentage of root length colonized by fungal hyphae among all plant species

577 for each fungal inocula. Control and Wash treatments are included for reference only.

578 Letters indicate significant differences between fungal treatments ($p<0.0001$). Fungal

579 inocula and figure symbols are as in Figure 2.