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Mycorrhiza

Native arbuscular mycorrhizal symbiosis alters foliar bacterial community composition

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Dear Editor,

Please find attached our second revised version of the manuscript titled "Native arbuscular mycorrhizal symbiosis alters foliar bacterial community composition" by Anbu Poosakkannu, Riitta Nissinen, and Minna-Maarit Kytöviita. We thank you for your valuable comments and time. I carefully looked at your comments and I accepted almost all of them. Wherever applicable we used the term AM fungus to make sure we studied only one species. The primer name fITS7 is correct (We used same as in original reference). I have also modified the table legends and figures according to the suggestions. We hope the current version can be accepted for the publication.

Thanking you.

Sincerely yours,

Anbu Poosakkannu

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Native arbuscular mycorrhizal symbiosis alters foliar bacterial community composition

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Abstract

The effects of arbuscular mycorrhizal (AM) fungi on plant-associated microbes are poorly known. We tested the hypothesis that colonization by an AM fungus affects microbial species richness and microbial community composition of host plant tissues. We grew the grass *Deschampsia flexuosa* in a greenhouse with or without the native AM fungus, *Claroideoglomus etunicatum*. We divided clonally produced tillers into two parts: one inoculated with AM fungus spores and one without AM fungus inoculation (non-mycorrhizal, NM). We characterized bacterial (16S rRNA genes) and fungal communities (internal transcribed spacer region) in surface-sterilized leaf and root plant compartments. AM fungus inoculation did not affect microbial species richness or diversity indices in leaves or roots, but the AM fungus inoculation significantly affected bacterial community composition in leaves. A total of three OTUs in leaves belonging to the phylum Firmicutes positively responded to the presence of the AM fungus in roots. Another six OTUs belonging to the Proteobacteria (Alpha, Beta and Gamma) and Bacteroidetes were significantly more abundant in NM plants when compared to AM fungus-inoculated plants. Further, there was a significant correlation between plant dry weight and leaf microbial community compositional shift. Also, there was a significant correlation between leaf bacterial community compositional shift and foliar nitrogen content changes due to AM fungus inoculation. The results suggest that AM fungus colonization in roots has a profound effect on plant physiology that is reflected in leaf bacterial community composition.

Keywords

Arbuscular mycorrhizal fungi; plant-associated microbes; high-throughput sequencing; foliar nitrogen

Introduction

All plants are associated with microbes, and today the importance of microbes for plant performance is well acknowledged. Mycorrhizal symbiosis, nodule forming bacteria and pathogens affect diverse plant species, and these symbiotic systems are relatively well studied. Recently, however, it has become clear that the symbiotic systems are complex in nature and multiple microbial interactions shape plant performance (Faust and Raes 2012). For instance, taxonomically diverse bacteria affect mycorrhizal fungal colonization of host plants and are known as 'mycorrhiza helper bacteria' (Frey-Klett et al. 2007; Bonfante and Anca 2009; Xavier and Germida 2003; Duponnois and Plenchette 2003).

The microbes that live in close association with plants are known as plant-associated microbes. The major plant-associated microbes are present in the phyllosphere (leaf surface), rhizosphere (root-attached soil) and endosphere (inside plant). Plant-associated microbes either are acquired from the environment or are passed from one generation to the next through propagules (Hardoim et al. 2008; Ferreira et al. 2008; Hardoim et al. 2012). Many of these microbes have neutral or positive impacts on host plants (Hansen and Moran 2014; Haney et al. 2015, Ludwig-Müller 2015, Panke-Buisse et al. 2015). Distinct factors such as plant part inhabited, host genotype and soil type affect the plant-associated microbial assemblage (Lundberg et al. 2012; Edwards et al. 2015; Coleman-Derr et al. 2016; Robinson et al. 2016; Poosakkannu et al. 2015; Poosakkannu et al. 2017).

Arbuscular mycorrhizal fungi (AMF) inhabit the plant root endosphere and rhizosphere. The ecological influence of AMF is partly due to their hyphal network that connects the

inside of the host plants to the soil (Miller et al. 1995). AMF may improve host plant nutrient and water uptake (Finlay 2008). In return, AMF utilize photosynthetically assimilated carbohydrates from the host plants (Bago et al. 2000). AMF symbiosis is accompanied by several alterations in gene expression in the roots and shoots of mycorrhizal plants which are related to a variety of plant physiological functions (Liu et al. 2007).

As AMF have fundamental effects on both shoot and root physiology, it is conceivable that the symbiosis with AMF may affect other host-associated fungal and bacterial communities. Nevertheless, the effect of AMF on plant-associated microbial communities is poorly studied. It has been shown that AMF inoculation can positively or negatively affect leaf and root endophytic fungal community composition (Eschen et al. 2010; Wearn et al. 2012; Larimer et al. 2012). Also, in return, endophytic fungi could affect AMF colonization rate (Larimer et al. 2012). In contrast to interactions between AMF and endophytic fungi, thus far AMF effects have been verified on bacterial communities in soil (Scheublin et al. 2010, Wamberg et al. 2003; Gryndler 2000; Jeffries et al. 2003) and in the rhizosphere (Marschner et al. 2001; Scheffknecht et al. 2006; Gupta 2003; Vigo et al. 2000).

Plant physiological status is one of the main factors determining the colonization and compatibility of plant-associated microbes (Gaiero et al. 2013). It is very well known that the presence of AMF increases host phosphorous and nitrogen contents (Karandashov and Bucher 2005; Fellbaum et al. 2012; Hodge and Fitter 2010). Recently, it has been shown that the rhizosphere bacterial community composition changes induced by AMF inoculation are related to changes in plant physiology, for

instance, changes in plant phosphorus content (Rodríguez et al. 2017). Similarly plant physiological changes due to AMF inoculation could affect other plant-associated microbes.

It has been shown that AMF hyphae and spores harbor diverse microbial species (Bonfante and Anca 2009; Selvakumar et al. 2016). Also, it is known that different bacterial and fungal species co-occur with AMF (Mansfeld-Giese et al. 2002). Therefore, AMF-associated microbes could be a source for plant-associated microbial consortia and subsequently might affect the microbial species richness of host plants. In order to gain insight into the interactions between AMF and other plant-associated microbes, we asked the following research questions: (i) does inoculation with an arbuscular mycorrhizal (AM) fungus affect leaf and root microbial community composition; (ii) does foliar nitrogen content correlate with microbial community composition; and (iii) do AM fungus-colonized plants harbour more numerous microbial species in leaves and roots than non-colonized plants? We examined microbial community composition by high-throughput sequencing of 16S rRNA (bacteria) genes and internal transcribed spacer (ITS) regions (fungi). We manipulated AM fungus presence in host plants grown in sterilized substrate under greenhouse conditions. As model species, we used the wild circumpolar grass, *Deschampsia flexuosa* which is considered to be a keystone pioneer plant species in inland sand dune ecosystems in northern Finland (Poosakkannu et al. 2015; Poosakkannu et al. 2016) and a native AM fungus species, *Claroideoglomus etunicatum*.

Materials and methods

Study organisms

122 *Deschampsia flexuosa* is commonly known as wavy hair grass, a cosmopolitan species
123 of temperate and subarctic regions (Scurfield 1954). Seeds of *D. flexuosa* were collected
124 from inland sand dunes in Northern Fennoscandia (68° 29' 16" N, 24° 42' 13" E) in
125 summer, 2012. The seeds were germinated in sterile sand and grown under greenhouse
126 conditions by providing water and Ingestad nutrient solution (Ingestad 1979).
127
128 *Claroideoglomus etunicatum* isolate was prepared from arctic sand dune soil by first
129 proliferating it in pot culture among mixed AMF species, followed by growing a hand-
130 picked single spore and later a mass inoculum in sterilized growth substrate with
131 *Plantago lanceolata* as the host. *C. etunicatum* has been isolated previously in different
132 ecosystems from Arctic tundra to tropical regions (http://invam.wvu.edu/the-
133 fungi/classification/claroideoglomeraceae/claroideoglomus/etunicatum; Toomer et al.
134 2015; Gupta et al. 2014), suggesting that it is a widely distributed species.
135
136 **Mycorrhizal inoculation and sampling**
137 *Deschampsia flexuosa* grows by clonally reproduced tillers and therefore the plants are
138 easily divided into genetically identical parts. In our experiment, each of the six
139 different pre-grown plants was divided into three parts. The fresh weights of all three
140 parts were measured (initial value). One part was planted into pots filled with substrate
141 (A 9:1 mixture of sterile sand and perlite with 1 g/L dolomite and 1 g/L bone meal) and
142 inoculated with 250 spores of *C. etunicatum*. The second part was planted in the same
143 substrate and mock inoculated with filtered AM fungus inoculation solution containing
144 no AM fungus spores as a control (NM, non-mycorrhizal plants). The third part was
145 used to determine the dry weights of the initial plant samples.
146

The AM fungus inoculum for the present experiment was prepared by soaking the substrate containing spores in water for 2 hours, agitating the suspension vigorously, letting the solution sediment for approximately 5 seconds followed by decanting. The decanted solution containing the spores was used as the AM fungus inoculum. The NM control solution was prepared by additionally filtering the decanted AM fungus inoculum through a regular coffee filter. The filtrate was left to sediment for 2 minutes and decanted. The decanted solution was checked to confirm the absence of any AM fungus spores or hyphae, but was assumed to contain representatives of other microbial community members which accompanied the inoculum. The AM fungus and NM inoculations were applied as 6.4 ml pipetted suspensions onto the growth substrate and plant roots. The experiment was started on 1 May, 2014, and a total of six replicates of NM and AM fungus inoculation treatments each were maintained during the experiment under greenhouse conditions. The plants were watered once a day and fertilized with Ingestad nutrient solution containing 0.9 mM nitrogen and 0.06 mM phosphorous twice a week (Ingestad 1979).

The plants were harvested on 15 October, 2014. The shoot and root dry mass (final values) were measured. Leaves (250 mg) and roots (250 mg) from each plant (3 to 4 representative leaves or roots for each plant) were surface sterilized following the method described in Poosakkannu et al. (2015) for molecular analysis. In brief, leaves and roots of each plant were surface sterilized by soaking in 70 % ethanol for 1 min, in 3 % sodium hypochlorite for 3 minutes, and in 1 % sodium thiosulphate for 3 minutes, followed by a series of three washes with sterile deionized water for 3 minutes each. For determination of AM fungus colonization, a portion of roots was stored in 50 % ethanol until examined for colonization.

172

173 **Fungal colonization measurements**

174 Roots from each individual were stained after clearing by incubation in 5 % KOH
175 overnight. After 2 h in 1 % HCl, the roots were stained with 0.02 % trypan blue solution
176 for 2 h at 80 ºC. The AM fungal root colonization was measured at ×100 magnification
177 from 10 root segments of 1.5 cm length using the cross-hair intersection method
178 (McGonigle et al. 1990). A total of 100 intersections were assessed per root sample.

179

180 **Foliar nitrogen content analyses**

181 Oven dried (65°C, 24h) leaf samples were ground to powder and samples (1.8 mg each)
182 were measured for total nitrogen using an elemental analyzer (Flash EA1112, Carlo
183 Erba) connected to a Finnigan Deltaplus Advantage (Thermo Electron Corp., Waltham,
184 USA) continuous flow isotope ratio mass spectrometer (CFIRMS). We calculated the
185 nitrogen content of the plants using following formula: mg nitrogen/plant shoot=
186 (percentage nitrogen shoot /100) × dry weight mg shoot.

187

188 **DNA extraction, library preparation and sequencing**

189 The surface sterilized leaves and roots of each plant were frozen using liquid nitrogen,
190 ground and homogenized. DNA was extracted from the homogenized leaf (100 mg) and
191 root (100 mg) material from each plant using Invisorb Spin Plant Mini Kit (Stratec
192 molecular). We used the M13 system (Mäki et al. 2016) for library preparation as
193 described by Poosakkannu et al. (2017). In brief, a nested approach was used to amplify
194 the partial 16S rRNA genes; the first round of 16S rRNA amplification was performed
195 with primers 799F (5´-AACMGGATTAGATACCCKG-3´) and 1492R (5´-
196 GGYTACTTGTTACGACTT-3´) which excludes plant chloroplast amplification

(Chelius and Triplett 2001). The subsequent PCR was performed with M13-1062F (5´-FGTCAGCTCGTGYYGTGA-3´) and P1-1390R (5´-ACGGGCGGTGTGTRCAA-3´) primer pairs targeting the V7-V9 region. The ITS region was amplified using the fITS7 (5´-GTGARTCATCGAATCTTTG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) primer pairs (Ihrmark et al. 2012). We followed Ion PGM Sequencing 400 Hi-Q Kit manufacturer's instructions to carry out the sequencing (Ion 314 chips; Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Bioinformatics and statistics

The processing of bacterial 16SrRNA gene sequences was performed following a standard procedure in Mothur v.1.35.0 (Schloss et al. 2011). The parameters are minlength = 200; maxambigs = 0; maxhomop = 8; qwindowaverage = 25; qwindowsize = 50; and bdiffs = 1). The fungal sequences were processed as described by Tedersoo et al. (2015) using the same parameters as for the 16S rRNA genes. One of the AM fungus-inoculated root samples was not included in further downstream analyses because of not having enough good-quality sequences. The good-quality bacterial and fungal sequences were clustered at 97 % sequence similarity. Samples were normalized to their lowest number of reads for further statistical analyses. The observed species richness, estimated species richness (Chao1), Shannon and Inverse Simpson diversity indices were calculated using the normalized data. Raw data were submitted to the sequence read archive (SRA accession number: SRP087758).

The clustering pattern of bacterial and fungal community composition by treatment was visualized using unconstrained principal coordinate analysis (PCoAs) and the statistical significance of clustering patterns were estimated by Permutational Multivariate

Analysis of Variance (PERMANOVA; Anderson 2005). To test the correlation between microbial community composition and plant parameters (total, shoot and root dry weight and foliar nitrogen content), we performed distance-based linear model (DistLM) statistical analyses. All the above statistical analyses were performed in PRIMER software V6 (Clarke and Warwick 2001). Generalized linear model statistics were used to find the significance of diversity indices between treatments in R software (v 1.0.44). One-way analysis of variance was carried out for the plant biomass and foliar nitrogen content analyses after testing the homogeneity of variances using SPSS software (IBM SPSS 24).

Results

We obtained 94,836 and 45,628 good quality bacterial and fungal sequences, respectively. These sequences resulted in 2,300 bacterial OTUs and 1,460 fungal OTUs. We excluded OTUs with less than five sequences, which resulted in 487 (92,418 sequences) bacterial OTUs and 418 (43,925 sequences) fungal OTUs. In one sample, there were between 1168 and 7,541 bacterial sequences and between 220 and 1,920 fungal sequences. The normalization of samples resulted in 468 and 385 OTUs of bacteria and fungi. A total of 14 bacterial and 3 fungal phyla were found in the two compartments of the NM and AM fungus-inoculated plants (Figure 1). Because of the primers used, we were not able to detect the fungal phyla Glomeromycota and Zycomycota in this experiment (Figure 1B).

Effect of AM fungus inoculation on plant growth and foliar nitrogen content

At the end of the experiment, no AM fungal colonization was found in NM plants, while all the AM fungus-inoculated plants were colonized. The average hyphal colonization rate was 42 ± 6 %.

The total plant dry weight was significantly ($p<0.05$) higher for AM fungus-colonized plants (569.05 ± 195.71 mg) than for NM plants (211.33 ± 100.69 mg). Also, AM fungus inoculation significantly increased the foliar nitrogen content of AM fungus-colonized plants (7.6 ± 2.7 mg / plant shoot) versus NM plants (4.7 ± 1.7 mg / plant shoot).

Effect of AM fungus inoculation on bacteria

AM fungus inoculation did not affect the species richness (observed and estimated) or diversity indices (Shannon and Inverse Simpson) of bacteria in either leaf or root (Table 1). AM fungus and NM plant leaf bacteria clustered strongly according to the AM fungus inoculation treatment, and the first axis of the PCoAs explained 18.1% of the variation (Figure 2). The PERMANOVA analysis confirmed the significance of the AM fungus treatment on leaf bacterial community composition (Figure 2). In contrast to leaf bacteria, AM fungus colonization did not affect the root bacterial community composition (Figure 2). A total of 90% bacterial sequences were shared between NM and AM fungus-inoculated plants (Supplementary figures: Supplementary figure 1). They accounted for 47 % of the total bacterial OTUs.

In order to gain insight into the effect of AM fungus treatment on the relative abundance of individual OTUs, we performed Kruskal -Wallis tests with log transformed (log [X+1]) relative abundance data to identify the OTUs that are responsible for community

separation between the NM and AM fungus treatments. We considered only OTUs with greater than 1% relative abundance in at least three samples of leaves or roots. There were three bacterial OTUs that were statistically significantly ($p<0.05$) more abundant in AM fungus-inoculated plant than in NM plant leaves (Figure 3). As an example, these included the OTU 00006 in the bacterial family "Bacillaceae" belonging to the Firmicutes phylum. There were six bacterial OTUs statistically significantly ($p<0.05$) more abundant in NM than AM fungus-inoculated plant leaves (Figure 3). For instance, the OTU 00001 classified as the bacterial genus "*Pseudomonas*" belonging to the Proteobacteria phylum was significantly more abundant in NM than in AM fungus-inoculated leaves. In contrast, there was no significant difference in any root bacterial OTUs between NM and AM fungus-inoculated plants.

Effect of AM **fungus inoculation on fungi**

The AM fungus inoculation did not affect the species richness (observed and estimated), Shannon diversity and Inverse Simpson diversity of fungi in either leaves or roots (Table 1). Also, fungal community composition was not affected by the AM fungus inoculation (Figure 4). A total of 93 % fungal sequences were shared between NM and AM fungus-inoculated plants (Supplementary figures: Supplementary figure 1). They accounted for 55 % of total fungal OTUs. Furthermore, there were no significant differences in any leaf or root fungal OTUs between NM and AM fungus-inoculated plants.

Correlation between microbial community composition and plant parameters

Significant correlations were observed between the dry weights (Total, shoot and root) and leaf bacterial community composition ($p<0.05$; Table 2). Similarly, significant

295 correlations were observed between the plant dry weights (Total and shoot) and leaf
296 fungal community composition ($p<0.05$; Table 2).
297
298 Foliar nitrogen content and leaf bacterial community composition also were correlated
299 significantly ($p<0.05$; Table 2). The correlation explained 13% of the variation in leaf
300 bacterial community (Table 2). In contrast, there was no significant correlation observed
301 between foliar nitrogen content and leaf fungal community composition ($p>0.05$; Table
302 2). Also, there was no significant correlation between foliar nitrogen content and root
303 microbial community composition ($p>0.05$; Table 2).
304
305 **Discussion**
306 To our knowledge, this is the first study to consider the effect of AM fungus
307 colonization on foliar bacteria. In our study, the bacterial community composition in
308 *Deschampsia flexuosa* leaves but not roots was altered by arbuscular mycorrhiza
309 formation. Consistent with our study, Groten et al. (2015) showed that AM fungus
310 colonization did not affect the root-associated bacterial community composition of
311 *Nicotiana attenuata*.
312
313 In our study, phylogenetically diverse bacterial OTUs in the phyla Proteobacteria,
314 Firmicutes and Bacteroidetes were differentially regulated in the leaves of NM and AM
315 fungus-colonized plants. The presence of AMF has been shown to increase relative
316 abundance of the members of the phylum Firmicutes in soil (Nuccio et al. 2013).
317 Similar results for leaves were observed in this study and three OTUs classified as
318 *Unclassifed Bacillaceae* (OTU00006), *Paenibacillus* (OTU00020) and *Brevibacillus*
319 (OTU00028) belonging to Firmicutes were significantly more abundant in AM fungus-

inoculated plant leaves. The bacterial genera *Paenibacillus* and *Brevibacillus* are described as mycorrhiza helper bacteria which increase mycorrhizal colonization of roots (Frey-Klett et al. 2007). Also, these bacterial genera are known to be closely associated with mycelia of AMF (Mansfeld-Giese et al. 2002). The present results warrant further targeted experiments to understand whether the bacterial genera *Paenibacillus* and *Brevibacillus* could have an ecological function in the leaves of arbuscular mycorrhizal plants.

A total of six OTUs belonging to Proteobacteria (Alpha, Beta and Gamma) and Bacteroidetes were significantly more abundant in NM than in AM fungus-inoculated plant leaves. Notably, the most abundant OTU in this study belonging to the genus *Pseudomonas* (OTU00001) was significantly more abundant in NM than AM fungus-inoculated plant leaves. The genus *Pseudomonas* is well-known for denitrifying capacity and responds negatively to AMF presence in the rhizosphere and soils (Ravnskov et al. 1999; Amora-Lazcano et al. 1998; Meyer and Linderman 1986). The OTUs belonging to potential nitrogen fixing genera such as *Pseudomonas* and *Bradyrhizobium* were significantly more abundant in NM than in AM fungus-inoculated plant leaves. It has been shown that different bacteria genera and *Pseudomonas* in particular may fix atmospheric nitrogen in grass leaves (Bessems 1973; Pimentel et al., 1991). It also is known that many species of the bacterial genus *Pseudomonas* possess phosphate mobilization capacity in *D. flexuosa* plants (Poosakkannu et al. 2016). In contrast to our study, Ordoñez et al. (2016) showed that an AMF species (*Rhizophagus irregularis*) may have a synergistic effect with phosphate solubilizing *Pseudomonas* species. The more abundant *Pseudomonas* and other bacteria in NM than in AM fungus-

344 inoculated plants might fix atmospheric dinitrogen and mobilize phosphate for plant use
345 in the absence of efficient AM fungus-mediated nutrient transfer from soil.
346
347 In this study, the fungal community composition of *D. flexuosa* leaves and roots was not
348 altered by arbuscular mycorrhiza formation. This is in contrast with previous studies
349 which have shown that AMF colonization can affect the fungal community composition
350 of foliar and root fungi in different plant species such as *Cirsium arvense, Plantago*
351 *lanceolata* and *Rumex acetosa* (Eschen et al. 2010; Wearn et al. 2012). It is worth
352 noting that both NM and AM fungus-inoculated starting plant tillers in the present study
353 were of the same clone at the same plant growth stage and most likely contained similar
354 initial microbial communities which was not the case in previous, related studies
355 (Eschen et al. 2010; Wearn et al. 2012; Larimer et al. 2012). Plant genets may harbour
356 distinct microbial communities (Korkama et al. 2007), therefore the clone identity and
357 growing history should be accounted. The effect of AMF on microbial community
358 composition has been shown to be plant and AM fungus species dependent (Söderberg
359 et al. 2002; Larimer et al. 2012). The host plant and AM fungus species of the present
360 study are additional potential reasons for the difference in the effect of mycorrhizas on
361 fungal community composition observed between our study and earlier studies. Also,
362 the plants in this experiment were grown in sterilized substrate and the major source of
363 microbes may have been seeds, AM fungus and NM inocula, water or air. Another
364 explanation for differences may be the choice of methods. Eschen et al. (2010) and
365 Wearn et al. (2012) relied on culture methods for microbe detection in contrast to our
366 high-throughput sequencing. It is well known that culture methods and high-throughput
367 sequencing give different results (Zhang and Xu 2008; Van Elsas and Boersma 2011).
368

Plant mass and microbial community composition correlated significantly in our experiment. This is in agreement with reports that have shown that rhizosphere bacterial community composition may correlate with plant biomass (Rodríguez et al. 2017). In the present study, a significant correlation also was observed between bacterial community composition and foliar nitrogen content. In contrast, shoot nitrogen content did not correlate with rhizosphere bacterial community composition in the study by Rodríguez et al. 2017. The difference between the studies could be due to the compartments studied (rhizosphere soil vs surface-sterilized tissues). We did not measure any other foliar elements than nitrogen in this study, and it is possible that other nutrients mediated by AM fungus affected the foliar bacterial community. Furthermore, we cannot rule out the possibility that AM fungus-inoculated plants possibly provided more carbon compounds to foliar bacteria because of high net photosynthesis. Although we did not measure photosynthesis rates, we previously have shown that AMF-inoculated arctic plants with higher foliar nitrogen content than NM plants also photosynthesize at higher rates (Ruotsalainen & Kytöviita 2004). The current study together with that by Rodríguez et al. (2017) shows that the mycorrhizal status of plants alters plant physiology and subsequently could change the community composition of bacteria intimately associated with plants.

In our study, bacterial and fungal species richness and diversity indices were not affected by the presence of an AM fungus in either leaves or roots of *D. flexuosa*. This is consistent with an earlier finding that non-mycorrhizal transgenic *Nicotiana attenuata* plants harbour similar root-associated fungal and bacterial species richness to that of mycorrhizal transgenic *N. attenuata* plants (Groten et al. 2015). Likewise, inoculation with AMF has been shown not to alter bacterial species richness in the rhizosphere

(Rodríguez et al. 2017). Furthermore, while AMF spores harbour microbes (Selvakumar et al. 2016), in our study, most microbial sequences were shared between NM and AM fungus-inoculated plants. This suggests that mock inoculation of spore-washing water to NM plants most likely transferred at least part of AM fungus-associated microbes.

In conclusion, this study demonstrates that AM fungus inoculation can modify leaf microbial composition under semi-sterile greenhouse conditions. Further targeted studies are needed to understand the mechanisms involved and the functional aspects in the interaction between leaf microbes and AM fungus.

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Figure legends

Fig. 1. Phylum-level distribution based on (A) the bacterial 16S rRNA gene, and (B) fungal internal transcribed spacer region sequences of *Deschampsia flexuosa* leaf and root samples expressed as relative abundance in non-mycorrhizal and arbuscular mycorrhizal (AM) fungus-inoculated plants. Values mean of six (except for AM fungus-inoculated root fungi with five replicates) replicates from each sample. OTUs were clustered at 97 % similarity.

Fig. 2 Clustering pattern of bacterial (16S rRNA gene) community composition in *Deschampsia flexuosa* (A) leaves, and (B) roots in the non-mycorrhizal and arbuscular mycorrhizal fungus-inoculated plants. OTUs were clustered at 97 % similarity. The weighted and constrained principal-coordinate analysis (PCoA) based on Bray-Curtis dissimilarity on standardized and square-root transformed data was performed in PRIMER software v6. The pseudo-F and p values were obtained from PERMANOVA.

Fig. 3 Log-transformed relative abundances of the significantly different ($p<0.05$) bacterial (16S rRNA gene) OTUs in arbuscular mycorrhizal (AM) *Deschampsia flexuosa* leaves compared to non-mycorrhizal (NM) plant leaves. The number of replicates is six. Only OTUs with a relative abundance more than 1 % in at least three samples were included in the analysis. Three bacterial OTUs (OTU 00006, 00020, 00028 belonging to bacterial phylum, Firmicutes) were significantly more abundant in AM fungus-inoculated *D. flexuosa* leaves compared to NM *D. flexuosa* leaves. Another six bacterial OTUs (OTU 00001, 00010, 00017, 00033, 00035 belonging to phylum Proteobacteria and OTU 00046 belonging to bacterial phylum, Bacteroidetes) were

694 significantly more abundant in NM *D. flexuosa* leaves than in AM fungus-inoculated *D.*
695 *flexuosa* leaves.
696
697 Fig. 4 Clustering pattern of fungal (internal transcribed spacer region) community
698 composition in *Deschampsia flexuosa* (A) leaves, and (B) roots in the non-mycorrhizal
699 and arbuscular mycorrhizal fungus-inoculated plants. OTUs were clustered at 97 %
700 similarity. The weighted and constrained principal-coordinate analysis (PCoA) based on
701 Bray-Curtis dissimilarity on standardized and square-root transformed data was
702 performed in PRIMER software v6. The pseudo-F and p values were obtained from
703 PERMANOVA.
704

Figure1

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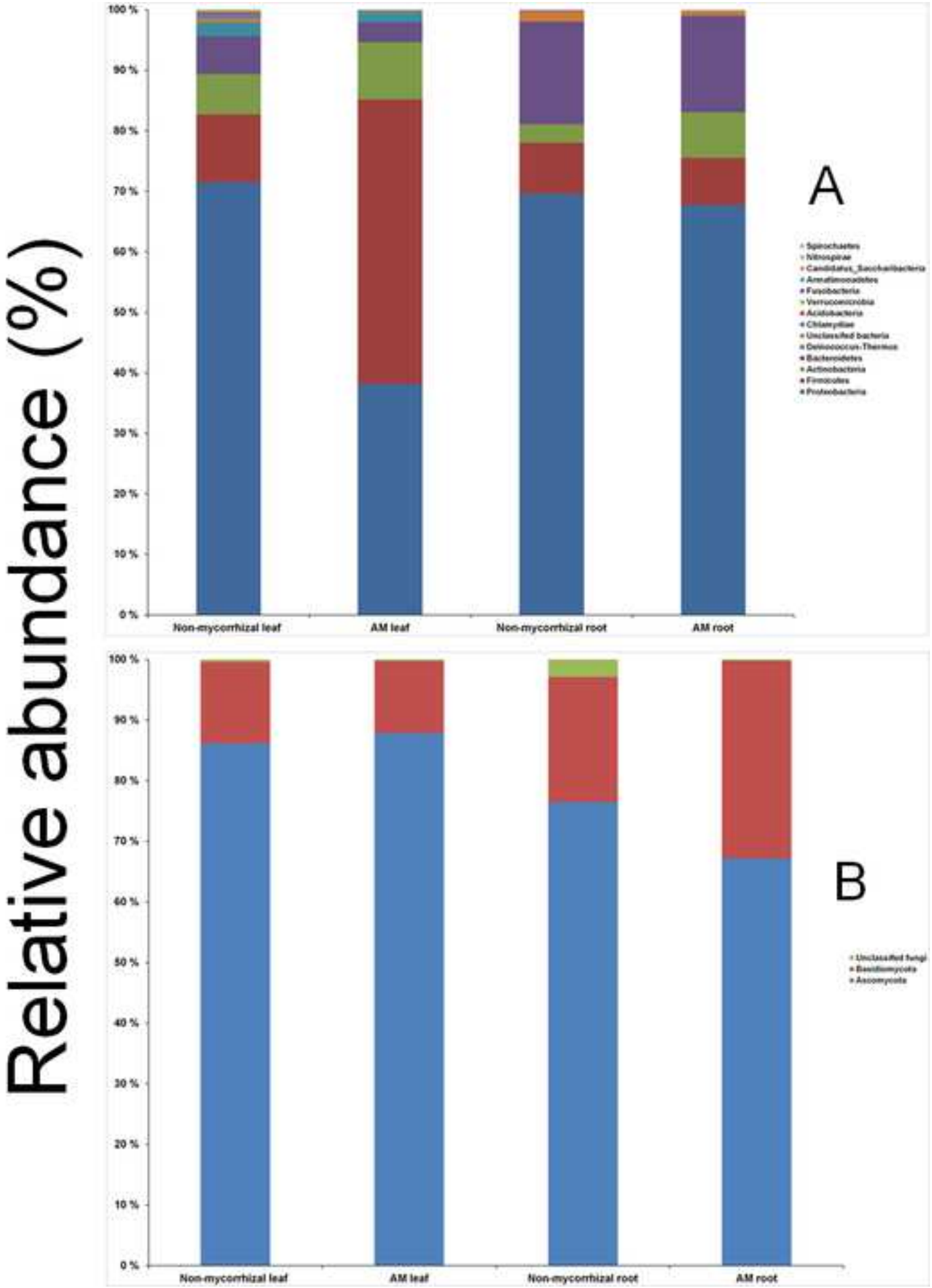

Figure2

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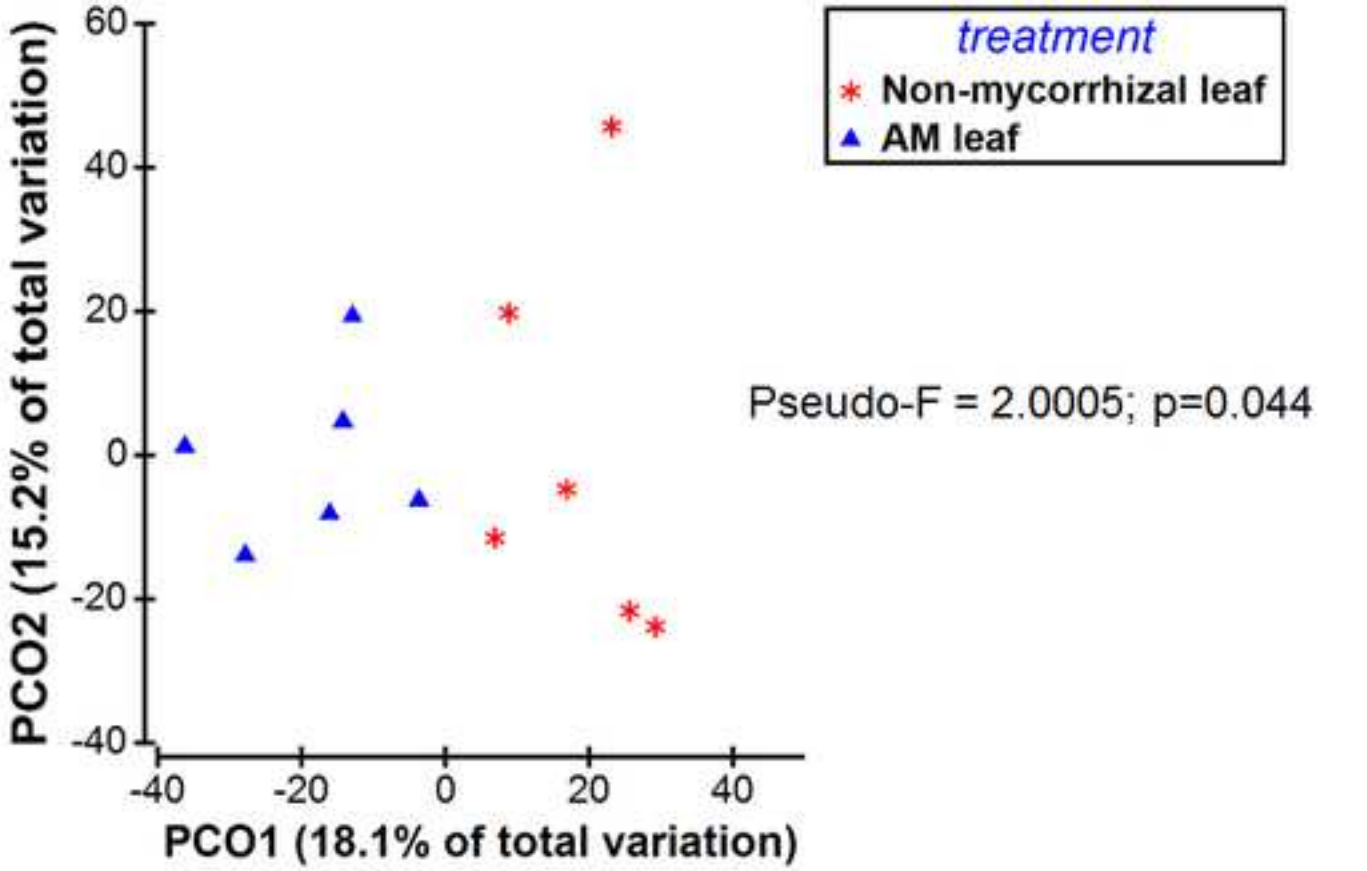

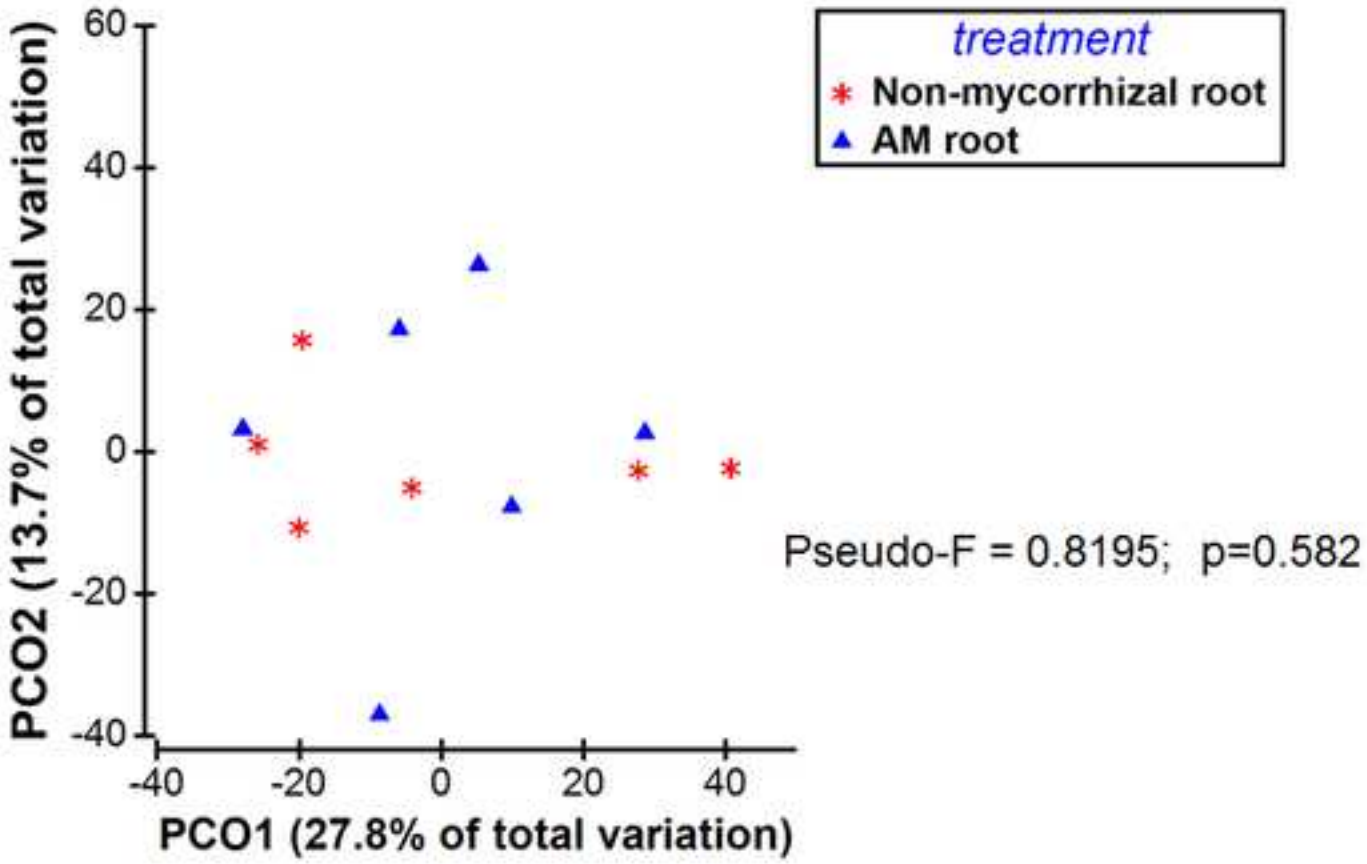

Figure3

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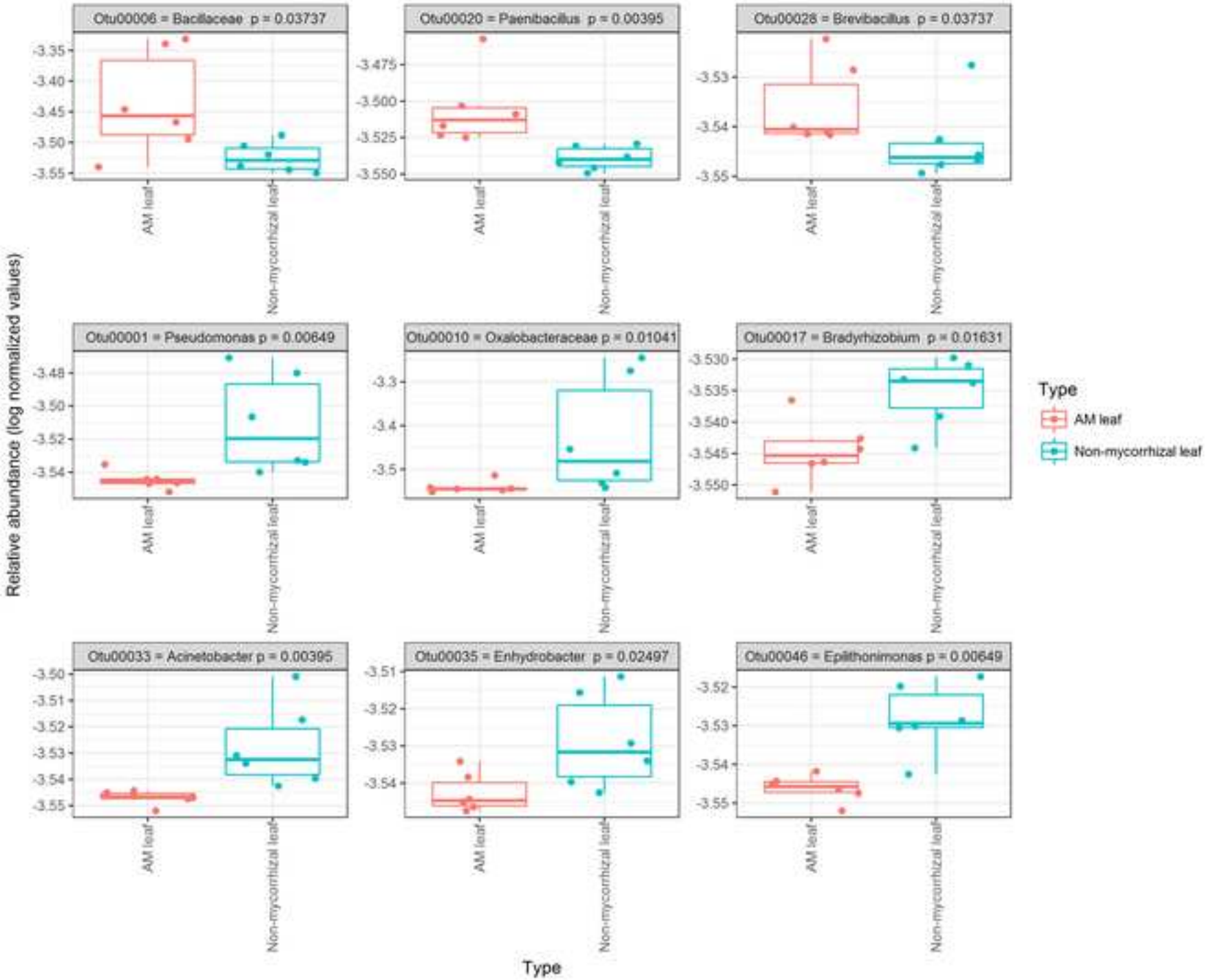

Figure4

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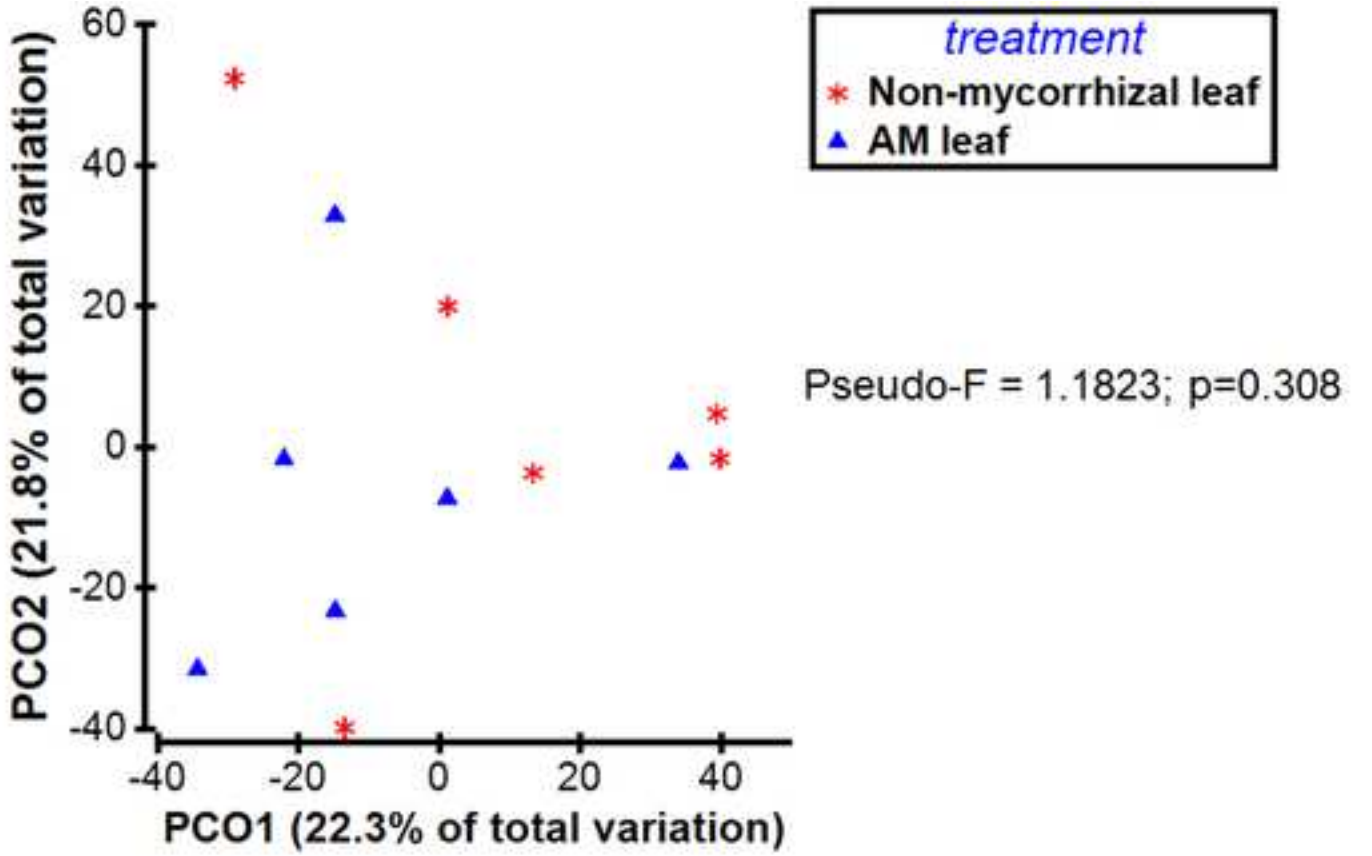

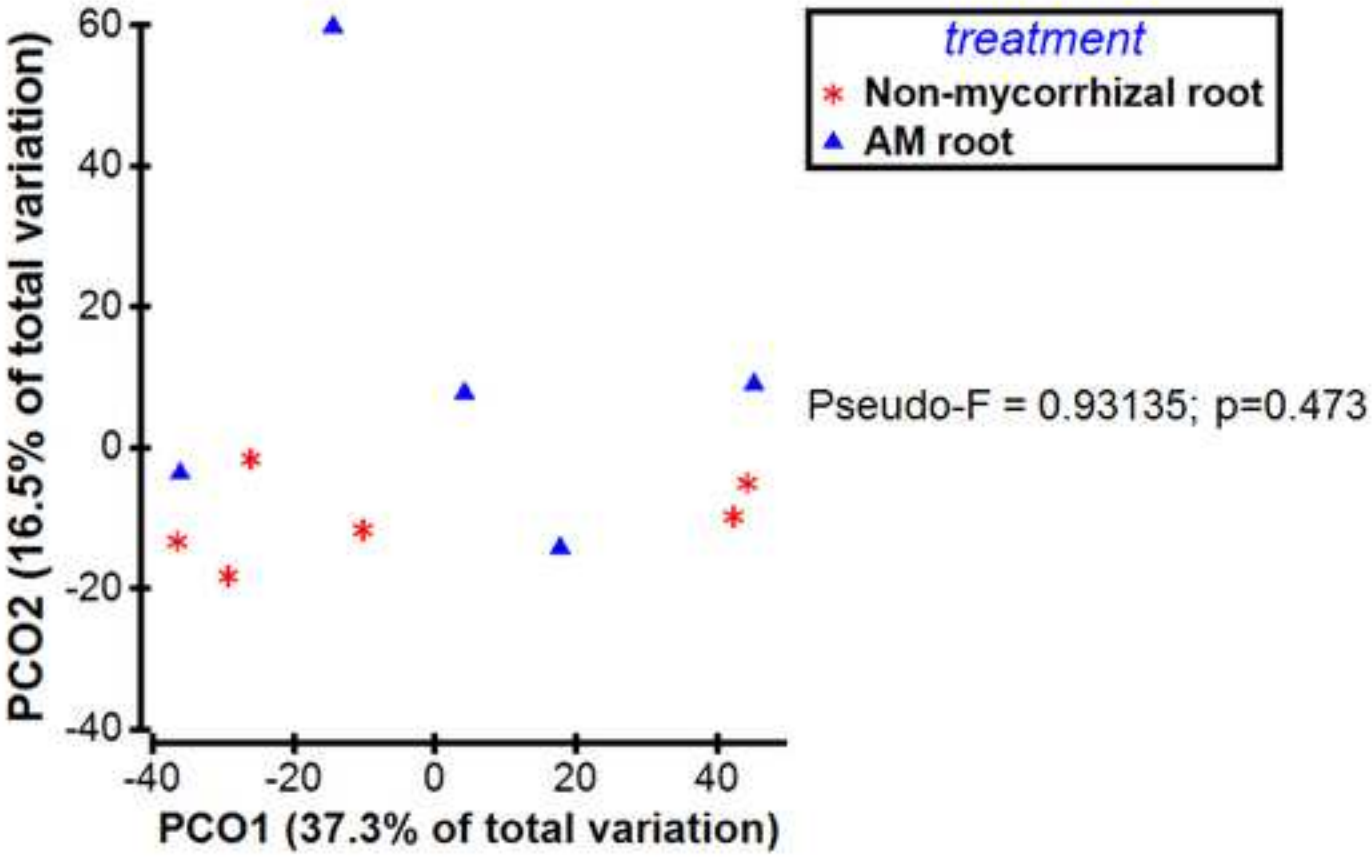

Taxa	Diversity indices	Treatment	Leaf (mean ± SE)	Root (mean ± SE)
Bacteria	Observed OTU richness	NM	62.50±7.01	87.67±11.57
		AM	59.17±5.70	75.67±5.38
	Estimated OTU richness	NM	81.03±14.86	122.15±15.90
		AM	76.37±8.24	107.56±5.31
	Shannon diversity index	NM	2.80±0.32	2.50±0.47
		AM	2.77±0.21	2.55±0.36
	Inverse Simpson index	NM	10.74±2.58	8.16±2.33
		AM	9.12±2.30	8.65±2.84
Fungi	Observed OTU richness	NM	42.33±7.49	30.17±2.57
		AM	38.50±6.21	36.20±6.70
	Estimated OTU richness	NM	62.44±8.32	46.39±6.12
		AM	75.62±11.53	57.48±15.27
	Shannon diversity index	NM	2.51±0.38	2.23±0.09
		AM	2.16±0.29	2.28±0.30
	Inverse Simpson index	NM	10.79±4.51	5.73±0.74
		AM	4.72±0.95	6.06±1.43

Table 1. Observed OTU richness, estimated OTU richness, Shannon-wiener index and Inverse Simpson index of bacterial OTUs (16S rRNA gene) and fungal OTUs (internal transcribed spacer region) in *Deschampsia flexuosa* leaf and root in non-mycorrhizal (NM) and arbuscular mycorrhizal (AM) fungus-colonized plants. OTUs were clustered at 97 % similarity level. Generalized linear model analyses showed that none of the differences are statistically significant between NM and AM fungus-colonized plants.

Table2

(A) Leaf bacteria				
Source of variation	**SS(trace)**	**Pseudo-F**	**P**	**Proportion**
Total weight	**4419.7**	**1.8177**	**0.002**	**0.15381**
Shoot weight	**4179**	**1.7018**	**0.006**	**0.14543**
Root weight	**4654**	**1.9327**	**0.001**	**0.16197**
Foliar nitrogen	**3821.5**	**1.5339**	**0.021**	**0.13299**
(B) Root bacteria				
Source of variation	**SS(trace)**	**Pseudo-F**	**P**	**Proportion**
Total weight	1480.5	0.77018	0.764	7.15E-02
Shoot weight	1521.4	0.79316	0.742	7.35E-02
Root weight	1493.8	0.77765	0.745	7.22E-02
Foliar nitrogen	2406.2	1.3151	0.156	0.11622
(C) Leaf Fungi				
Source of variation	**SS(trace)**	**Pseudo-F**	**P**	**Proportion**
Total weight	**5464**	**1.9097**	**0.018**	**0.16035**
Shoot weight	**5465.8**	**1.9104**	**0.018**	**0.1604**
Root weight	4676	1.5905	0.092	0.13722
Foliar nitrogen	4841.9	1.6563	0.066	0.14209
(D) Root fungi				
Source of variation	**SS(trace)**	**Pseudo-F**	**P**	**Proportion**
Total weight	4081.1	1.5041	0.136	0.14319
Shoot weight	4097.4	1.5111	0.151	0.14376
Root weight	3605	1.3032	0.246	0.12649
Foliar nitrogen	4632.6	1.7468	0.088	0.16254

Table 2. Distance based linear model statistical test (DistLM) for correlation between microbial community composition and plant parameters (total, shoot, root dry weight and foliar nitrogen content) in *Deschampsia flexuosa* leaves (A and C) and roots (B and D). Bold letters indicate the significant correlation ($p<0.05$).