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SYMBIOSIS BETWEEN TALL FESCUE AND A FUNGAL SHOOT ENDOPHYTE
AFFECTS SOIL MICROBIAL COMMUNITIES

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Abstract

The symbiosis between tall fescue (*Festuca arundinaceum*) and a shoot-specific fungal endophyte (*Neotyphodium coenophialum*) has been relatively well studied but little attention has been given to how this relationship may impact the soil microbial community. Understanding how the symbiosis may structure soil microbial communities is important for understanding the cascade of effects that this symbiosis can have on belowground ecosystems. We used high-throughput DNA sequencing of selected microbial genes (the 16S rRNA gene and fungal ITS rRNA region) to examine bacterial and fungal microbial communities in the soil, respectively, to address the following questions: (1) How do the microbial communities differ between rhizosphere and bulk soil in a tall fescue grassland? (2) How are belowground microbial communities affected by the presence of various strains of endophyte *N. coenophialum*? We found that rhizosphere and bulk soils harbored distinct microbial communities, with rhizosphere communities containing significantly higher relative abundances of Bacteroidetes, α -Proteobacteria, β -Proteobacteria, γ -proteobacteria, and Chytridiomycota, while bulk soil contained higher relative abundances of Verrucomicrobia, Acidobacteria, Firmicutes, and Zygomycota. We also found that endophyte presence significantly influenced rhizosphere microbial communities, with a greater effect on fungal versus bacterial communities. In particular, we observed an increased relative abundance of root-associated (arbuscular mycorrhizal) fungi in fescue plants containing shoot fungal endophytes. Our data suggests a complex, tripartite interaction between shoot endophytes, tall fescue and root associated fungi, which could have greater implications for grassland soils.

Introduction

Tall fescue (*Festuca arundinaceum*) is a widely used forage grass for grazing livestock in the United States, covering an estimated 15 million hectares throughout the country (McNear, 2012). The prevalence of tall fescue is partly due to its symbiotic relationship with a shoot-specific fungal endophyte (*Neotyphodium coenophialum*) that grows throughout the plant shoots and consumes nutrients provided by the host plant while producing compounds that provide benefits to the host. These benefits include drought and heat tolerance (West, 1994; Marks & Clay, 1996), herbivory resistance (Elmi, West, Robbins, & Kirkpatrick, 2000), enhanced photosynthesis (Richardson, Hoveland, & Bacon, 1993; Marks & Clay, 1996; Newman et al., 2003), and nutrient-deficiency tolerance (Malinowski & Belesky, 2000).

Among the endophyte's exudates are ergot and loline alkaloids that deter both insect and mammalian herbivores (Fortier, Bard, Jansen, & Clay, 2000). Likewise, ergot alkaloids are a serious detriment to grazing livestock as it causes fescue toxicosis, a condition that adversely affects grazing behavior in cattle, decreasing weight gain and reproduction, which creates severe costs for the livestock industry (Stuedemann & Hoveland 1988, Hoveland, 1993). Ergot alkaloids produced by *N. coenophialum* also create health issues in horses, sheep, goats, and wild geese (Ryan et al., 2001; Christiansen, Hopper, Filipov, & Ryan, 2007; Burke, Jackson, & Robson, 2002; Smith, Rotstein, & Brownie, 2004; Conover & Messmer, 1996).

Due to the adverse effects on valuable livestock, in 2002, researchers inoculated tall fescue with a naturally occurring strain of *N. coenophialum*, or novel endophyte, that does not produce ergot alkaloids harmful to mammals. The novel endophyte still

produces loline alkaloids allowing it to retain its insect deterring properties while remaining more resistant to stressful abiotic conditions (Bouton et al., 2002). Since then, the commercial sale of tall fescue cultivars containing novel endophytes has begun alongside the eradication of fescue pastures containing the more common endophyte strain.

This transition of tall fescue cultivars represents a potentially major alteration of a 15 million hectare ecosystem and thus deserves more comprehensive research into the effects of these novel and common endophytes on ecosystem properties. Extensive research has been conducted on the tall fescue/endophyte interaction and its effect on herbivores, but relatively little attention has been given to how this interaction affects soil microbial community composition. It is important to understand how *N. coenophialum* interacts with soil microbes because of the major role of soil microbes in soil fertility and biogeochemical cycling. Furthermore, the relevance of this topic extends beyond tall fescue pastures, as grass ecosystems cover 20% of all land area on Earth (Schantz, 1954) and 20-30% of all grass species exhibit similar fungal endophyte symbioses with relatives of *N. coenophialum* (Leuchtman, 1992).

The objective of the present study was to examine the impact of the shoot-specific fungal endophyte *N. coenophialum* on soil bacteria and fungi. Our research questions included: (1) how do microbial communities differ between rhizosphere and bulk soil in a tall fescue grassland? And (2) how is soil microbial community composition affected by the presence of various strains of endophyte *N. coenophialum*? Previous research on leaves of tall fescue showed that leaf-surface microbial communities are significantly altered by endophytes due to loline alkaloid production (Roberts & Lindow, 2013). We

hypothesized that there would be a shift in soil microbial community composition with endophytes present, and that this shift varies among endophyte strains. In order to address our questions we used high-throughput DNA sequencing of the 16S rRNA gene and fungal ITS rRNA region to characterize bacterial and fungal microbe communities, respectively, of rhizosphere and bulk soils collected from experimental tall fescue plots.

Methods

Sample Collection

Soil samples were collected from an experimental tall fescue site in Kentucky, which was established in 2006 for a grazing preference experiment. The site was of uniform soil type and on a 2-3% slope. The site had 7 blocks, each with 8 different plots that varied either the tall fescue cultivar (97TF1 or PDF) or the endophyte status (no endophyte (E-), common endophyte (E+), and two novel endophytes (AR542 and AR584)). In order to better understand the plant/soil interaction, we sampled both rhizosphere soil (soil in direct contact with plant roots) and bulk soil (soil outside of the rhizosphere) at each plot. Samples were taken at the height of the plant-growing season and sent to the University of Colorado at Boulder, where they were stored at -20°C.

Sequencing and Data Processing

In order to assess diversity and composition of bacterial and fungal communities in each of the 112 soil samples, we used a DNA-sequencing-based approach. This first involved extracting the DNA from each of the 112 soil samples using the MoBio PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The DNA was then amplified following the approach described in Fierer et al. (2012). Briefly, a

polymerase chain reaction (PCR) was used to amplify DNA from two different gene markers to assess bacterial/archaeal, and fungal communities. To examine bacterial and archaeal communities, the V4 hypervariable region of the 16S rRNA gene was sequenced using error-correcting 12-bp barcoded primers (515f/806r). For fungal communities, the first internal transcribed spacer (ITS1) region of the rRNA operon was sequenced using ITS1-F/ITS2 barcoded primers. In both cases, samples were amplified in triplicate. Barcoded primers specific to each sample allowed for multiplexing of samples. PCR products from all samples were quantified using PicoGreen dsDNA assay, and pooled together in equimolar concentrations. Amplicons were cleaned and concentrated using the UltraClean PCR Clean-Up Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Samples were sequenced on an Illumina MiSeq instrument using the V2 300 cycle MiSeq kit (Illumina, Inc., San Diego, CA, USA) at the University of Colorado Next Generation Sequencing Facility, with separate runs for the 16S rRNA and ITS amplicon pools.

Sequences were processed using the UPARSE pipeline, which provides quality filtering of sequences (truncating sequences to 150-bp length, accounting for error in sequencing process, dereplicating and removing singleton sequences) and clustering of operational taxonomic units (OTUs) at the 97% sequence similarity threshold (Edgar, 2013). All representative sequences from the OTUs were discarded that were not $\geq 75\%$ similar to sequences contained in either the Greengenes 13_5 database (MacDonald et al., 2011) or UNITE November, 2012 database (Abarenkov et al., 2010) for 16S and ITS rRNA sequences, respectively.

Using the established representative sequences, raw (trimmed to 150-bp) sequences were categorized into OTUs at the 97% similarity threshold in order to

quantify the number of sequences representing each OTU per sample. The Ribosomal Database Project (RDP) classifier was used to classify bacterial/archaeal and fungal OTUs to taxonomic groups using the aforementioned databases. To compare all samples at equivalent sequencing depths, samples were rarefied to 4,000 and 250 sequences per sample for bacteria/archaea and fungi, respectively.

Statistical Analyses

To assess differences in microbial community composition, we first calculated Bray-Curtis dissimilarities among samples from square root transformed OTU counts using the *vegan* package in R (R Development Core Team, 2013). Differences in overall bacterial/archaeal and fungal community composition between multiple factors were assessed using permutational multivariate ANOVA (PERMANOVA) in Primer 6 (Clarke & Gorley, 2006). The PERMANOVA test was conducted using tall fescue cultivar, endophyte status, and bulk/rhizosphere sample type as fixed factors while block was used as a random factor. In order to understand which taxa differed between rhizosphere and bulk soil samples, paired t-tests were used to compare average relative abundance of main taxonomic groups, where each rhizosphere sample was paired with its corresponding bulk soil sample of the same block, tall fescue cultivar and endophyte status.

Pair-wise PERMANOVA was used to test for differences in community composition between each group of the endophyte factor, using PRIMER 6 (Clarke & Gorley). Paired t-tests were also used to identify which taxa were driving community differences among endophyte groups. Each sample was paired with its respective sample from a different endophyte group of the same block, tall fescue cultivar and soil type. All

t-tests were conducted in R (R Development Core Team), and a prior Shapiro-Wilks test was used to verify normal distribution of data.

Results

Results of the PERMANOVA analysis reveal that all factors significantly influenced ($P < 0.05$) overall community composition of bacteria/archaea and fungi (Table 1). The differences in soil type are displayed in figure 1. Block factor, which was treated as a random factor, had a significant effect on both bacteria/archaea and fungi. The components of variation shown in table 1 show that tall fescue cultivar and endophyte status were less influential than soil type and block, but still substantially affected bacteria/archaea and fungi.

To answer our first question and understand differences in microbial community composition between bulk and rhizosphere soils, samples were analyzed at the phylum and class level. Main bacterial taxa (taxonomic groups that composed a mean of $\geq 3\%$ of sequences) are shown in figure 1. Among bacteria, the most prominent phylum was Verrucomicrobia with significantly higher relative abundance in bulk soil versus rhizosphere ($P = 0.01$). Other main taxonomic groups were Bacteroidetes, Actinobacteria, Acidobacteria, Firmicutes, α -Proteobacteria, β -Proteobacteria, and γ -proteobacteria. Relative abundance of Acidobacteria ($P = 0.009$) and Firmicutes was significantly greater in bulk soil ($P = 0.001$), while Bacteroidetes, α -Proteobacteria, β -Proteobacteria, and γ -proteobacteria were significantly more abundant in the rhizosphere ($P = 0.000$ for each). The most prominent fungal phylum was Ascomycota. Other main fungal phyla were Zygomycota, Basidiomycota, Glomeromycota, and Chytridiomycota.

While zygomycota were significantly more abundant in bulk soils ($P = 0.022$), chytridiomycota were more abundant in the rhizosphere ($P = 0.001$)

To address our second question regarding the effect of endophytes on soil communities, a pairwise PERMANOVA was used to reveal significant variation in community composition among endophyte groups (Table 2). Bacterial/archaeal communities differed somewhat between endophyte groups E+ and AR542 and between AR542 and AR584. In addition, there was a strong difference between the endophyte control group, E-, and each endophyte group, E+, AR542 and AR584 ($P = 0.001$ for each comparison). Fungal communities also differed significantly between the E+ and AR542 groups ($P = 0.02$).

To determine which taxa were driving differences in fungal communities between endophyte groups, we examined how relative abundances of each taxonomic group differed between the E- group and each endophyte treatment (only taxonomic groups with > 1% average relative abundance were considered and analysis was conducted at the order level when family level information was unavailable). The only taxonomic group that showed a significant effect in rhizosphere samples was of the order Paraglomerales. Presence of Paraglomerales in rhizosphere samples increased with the presence of all endophyte types (Figure 2). Paired t-tests showed that common endophyte (E+) and novel endophyte (AR542) were both significantly associated with an increased presence ($P = 0.003$ and 0.002 , respectively) of Paraglomerales compared to control group (E-).

Discussion

Our results show that community composition differs between bulk soil and

rhizosphere samples. The rhizosphere is in direct contact with plant roots and is affected by plant respiration and nutrient exudation. Copiotrophic microorganisms, found in nutrient-rich environments, thrive here because of the high content of carbohydrates, amino acids and organic acids (Sørensen, 1997), while oligotrophic organisms, found in low-nutrient environments, tend to be more prominent in bulk soil. We found significantly more bacteria belonging to the taxonomic groups β -Proteobacteria and Bacteroidetes in the rhizosphere, while there were relatively more bacteria belonging to Acidobacteria in the bulk soil (Figure 1). This is consistent with previous studies on contrasting bacterial communities in bulk soil and rhizosphere (Fierer, Bradford, & Jackson, 2007; Axelrood et al., 2002; McCaig, Glover, & Prosser, 1999). In particular, Fierer et al. (2007) established that, while there is variation within phylum, β -Proteobacteria and Bacteroidetes generally consist of copiotrophic species, while Acidobacteria contains mainly oligotrophic species. Fungal communities also differed significantly, with greater relative abundance of Chytridiomycota in the rhizosphere and greater Zygomycota in bulk soil. Less research has been conducted on nutrient preferences of fungal groups. The differences we observed may be due to a difference in available nutrients or other factors.

The pair-wise PERMANOVA showed a significant and consistent difference in fungal communities among endophyte groups. Each endophyte treatment differed from the control endophyte group, and this difference was due largely to increased abundance of fungi belonging to the order Paraglomerales in rhizosphere samples. The fact that Paraglomerales were significantly affected is of great importance because all members of this order are arbuscular mycorrhizal fungi (AMF). AMF create a symbiosis with tall

fescue roots, as they do with 80% of terrestrial plants (Schüßler, Schwarzott, & Walker, 2001). While in rare cases, AMF symbionts act antagonistically, these fungi most often act as mutualists, providing macro- and micronutrients to the host, while receiving organic carbon from root exudates (Smith & Read, 1996). Our finding is consistent with this fact in that we only saw an AMF response to the endophytes in the rhizosphere soils. Previous research established that plants form multiple mutualistic associations simultaneously (e.g. with plant, fungal endophyte, rhizobia and AMF), but how they interact is highly dependent on complex biotic and abiotic factors (Larimer, Bever, & Clay, 2010).

While substantial research is available on various multiple-factor on multiple plant synergies, the tripartite interaction between tall fescue, *N. coenophialum*, and AMF is poorly understood. Chu-Chou et al. (1992) and Mack & Rudgers (2008) showed that *N. coenophialum* can decrease mycorrhizal colonization of tall fescue roots. For perennial ryegrass (*Lolium perenne*), colonization of mycorrhizal fungi decreased with presence of foliar endophytes (also of the *Neotyphodium* genus), and varied significantly by phosphorus and sugar content, as well as ryegrass and endophyte cultivar (Liu et al., 2011). Clearly, our findings stand in contrast to these previously published studies as we found that endophyte presence, regardless of whether they produce ergot or loline alkaloids, increased relative abundance of AMF, which indicates greater colonization of AMF.

Although we observed significant effects of endophyte presence, our hypothesis that effects would vary between strains is refuted because relative abundance of AMF increased with both common and novel endophytes. Because of this, we speculate that

the interaction between endophytes and AMF is not due to differences in alkaloid production. Instead, this interaction is likely occurring due to changes in tall fescue physiology, allowing the host to provide more nutrients to its soil symbionts. This is consistent with a study conducted with a different grass, *Bromus setifolius*, which clearly demonstrated enhanced mycorrhizal colonization with the presence of *Neotyphodium sp.* (Novas, Cabral, & Godeas, 2005).

Although we cannot say with certainty, one possible explanation for the increase in AMF contrary to most studies may be due to the absence of environmental stressors in our experiment. The tall fescue in these plots did not experience grazing, drought, exceedingly high temperatures, or nutrient deficiency, which is where we would expect the endophyte to be most beneficial. Under adverse conditions, *N. coenophialum* has been shown to affect tall fescue root morphology and activity. Drought conditions stimulate formation of root hairs with increased length and decreased diameter to maximize surface area for water acquisition (West, 1994). Phosphorus deficiency produced the same effect in some experiments (Malinowsky, Brauer, & Belesky, 1999), and also changed root exudate production. Malinowsky and Belesky (2000) suggest that, under stressed conditions, endophytes may trigger defense mechanisms in tall fescue as if it were infected with pathogenic fungi, releasing resveratrol (an anti-fungal phenol and antioxidant) and chitinase (an enzyme that breaks down fungal cell walls) from the roots. If our tall fescue plot experienced environmental stressors, we might expect a negative interaction, as other studies have shown. Perhaps because water and nutrients were readily available, the tripartite relationship appeared mutualistic all around. The reason we see an increase in AMF instead of no difference may mean that, under favorable

conditions, tall fescue resources are abundant enough to be partitioned to both AMF and foliar endophytes.

While the presence of endophytes in tall fescue has the potential to increase soil C storage (Iqbal, Siegrist, Nelson, & McCulley, 2012), the role of AMF in C storage remains unclear. Little research has been conducted on AMF of tall fescue and the effects on C cycling. Research conducted on multispecies grass prairies showed a strong positive correlation between AMF hyphal abundance and C sequestration (Wilson, Rice, Springer, & Hartnett, 2009). Another study produced confounding results, showing that the presence of AMF in a grassland ecosystem increased decomposition of organic matter in soils under elevated CO₂ conditions, thus decreasing C sequestration (Cheng et al., 2012). Many other studies have been conducted in this field with similarly confounding results. The tall fescue/endophyte/AMF relationship could have important implications for belowground C cycling, but more research is necessary in order to understand this complex interaction.

Our study reveals that the tripartite interaction between tall fescue, foliar endophyte, and AMF can be cooperative. However, under what conditions this synergism takes place and its implications for ecosystem functioning remain unclear. In order to elucidate these ambiguities, future studies could explore how biotic and abiotic stressors affect the tripartite relationship, how the colonization of symbionts affects crop yield, and how the interaction between symbionts of tall fescue affects greater biogeochemical processes. It is crucial to conduct more research on this interaction because of the relevance of tall fescue to the U.S. economy, because of its applicability to grassland soils across the world, and because of the important role that these soils have on the

functioning of their ecosystems.

Appendix I – Tables

Table 1. The effect of soil type, tall fescue cultivar, endophyte status and block on microbial communities in Kentucky soil samples. Factors were assessed using a PERMANOVA. Each factor shows a significant effect on overall community composition ($P < 0.05$)

Factor (Type)	Bacteria/Archaea			Fungi		
	Pseudo-F	<i>P</i>	Component of variation	Pseudo-F	<i>P</i>	Component of variation
Soil type (Fixed)	3.80	0.001	7.0×10^{-3}	3.39	0.001	9.1×10^{-3}
Block (Random)	2.35	0.001	1.2×10^{-2}	2.41	0.001	1.9×10^{-2}
Tall fescue cultivar (Fixed)	1.20	0.022	4.9×10^{-4}	1.34	0.006	3.4×10^{-3}
Endophyte status (Fixed)	1.09	0.041	4.6×10^{-4}	1.45	0.001	1.3×10^{-3}

Table 2. The effect of different endophyte statuses on bacteria/archaeal and fungal communities in Kentucky soil samples. The test was conducted using a pair-wise PERMANOVA. Multiple pair-wise comparisons show significant effect ($P < 0.05$) of endophyte status on communities, primarily in fungal communities.

Endophyte groups	Bacteria/Archaea		Fungi	
	t	P	t	P
E-, E+	1.01	0.426	1.37	.001
E-, AR542	1.07	0.070	1.28	.001
E-, AR584	1.03	0.294	1.32	.001
E+, AR542	1.09	0.042	1.19	.015
E+, AR584	0.99	0.602	0.987	0.564
AR542, AR584	1.10	0.035	1.04	0.257

Appendix II – Figures

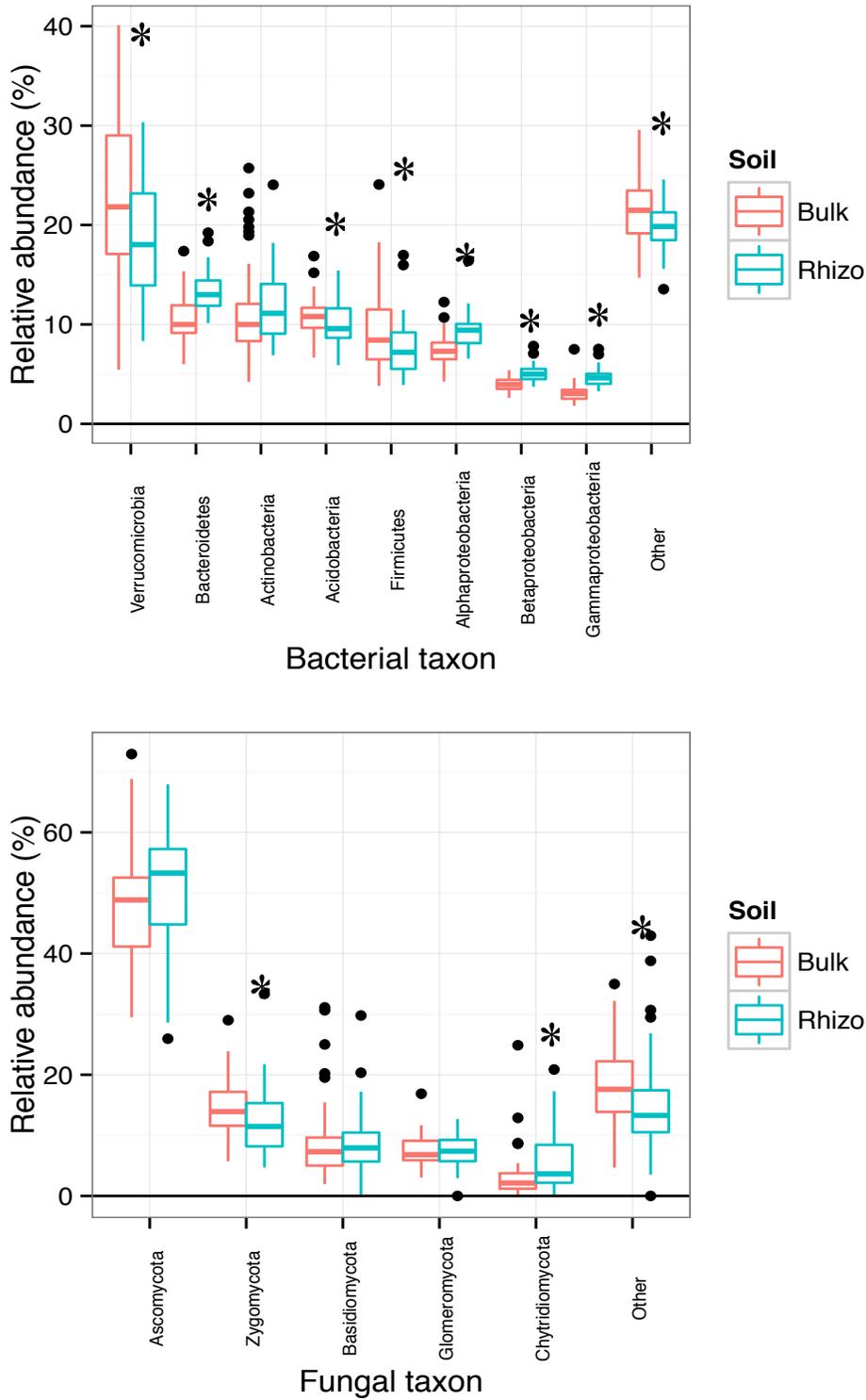


Figure 1. Boxplots showing the relative abundance (%) of main bacterial and fungal taxa, varied by soil type.

* indicates statistically significant difference ($P < 0.05$)

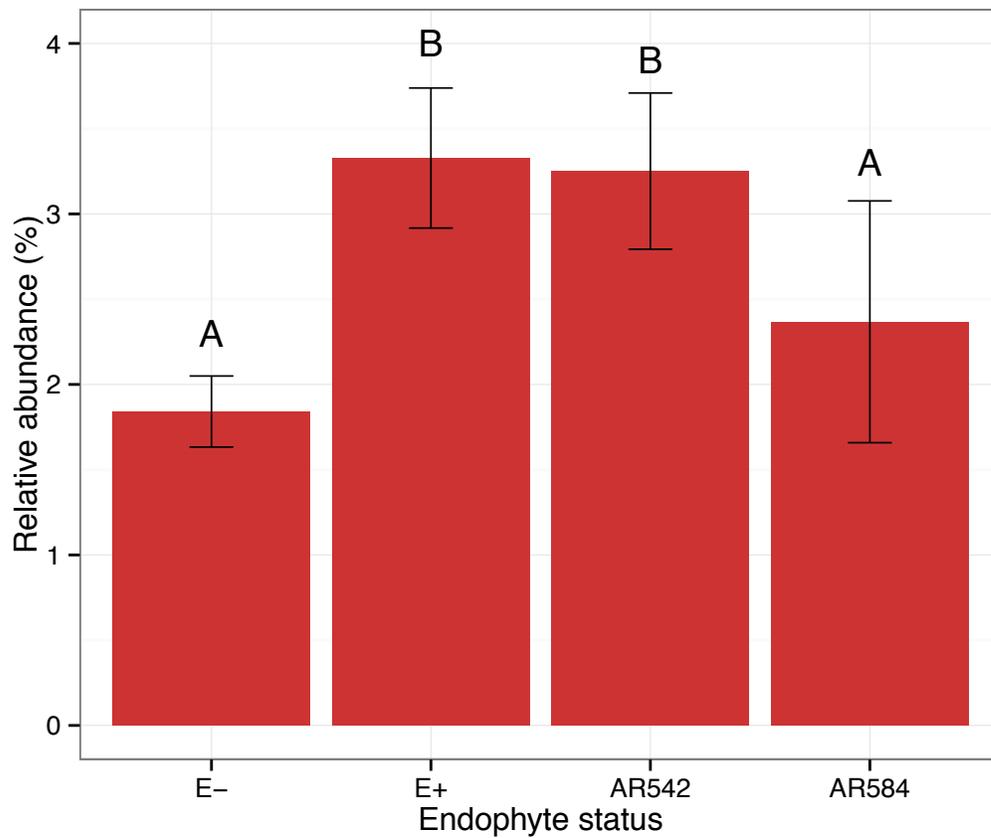


Figure 2. Mean relative abundances of fungi belonging to the order Paraglomerales (± 1 S.E.) for each endophyte treatment in only rhizosphere samples. Letters indicate statistical significance ($P < 0.05$).

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