Metabarcoding of the rhizosphere microbiome of perennial ryegrass in response to *Epichloë festucae* var. *lolii* infection

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Microbiology

At Massey University, Palmerston North

New Zealand

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2017

Abstract

Epichloë endophytes inhabit the intercellular spaces of cool-season pasture grasses, and can confer upon their hosts agriculturally desirable benefits such as heightened resistance to biotic and abiotic stresses. The mechanisms underlying many of these benefits are not well understood. Previously observed *Epichloë*-associated impacts towards the rhizosphere microbiome of their hosts could be a contributing factor, however the overall extent to which specific taxa in the rhizosphere microbiome of perennial ryegrass are affected by *Epichloë festucae* var. *lolii* infection remains to be elucidated. To assess this, two independent experiments were carried out in which clonal perennial ryegrass (NuiD) plants inoculated or uninoculated with *E. festucae* var. *lolii* (Lp19) originating from sterile tissue culture were grown in soil collected from a natural ryegrass pasture. After approximately two months of growth under controlled conditions in a growth cabinet, their prokaryotic and fungal rhizosphere microbiomes were compared using high-throughput metabarcoding.

For prokaryotes, endophyte infection had no significant impact on species richness or evenness of the rhizosphere microbiome of their hosts in either experiment. A very minor but significant shift in overall community composition was shown in the first experiment but not the second. At the level of phyla, aside from a minor 1.1% increase in the relative abundances of Bacteroidetes in the rhizosphere of infected compared with uninfected plants in the first experiment but not the second, there were no other significantly differentially abundant prokaryotic phyla due to endophyte infection. At the genus level rhizospheres of infected and uninfected plants showed a high degree of similarity in both experiments, with little variability between replicates within treatments. At the level of operational taxonomic units (OTUs), in the first experiment there was only one significantly differentially abundant OTU in the rhizosphere depending on endophyte infection, and nine in the second. However, all of which had relatively low abundances (<0.3%), and none were consistently significantly differentially abundant in both experiments.

For fungi, there were no significant impacts of endophyte infection on species richness or evenness of the rhizosphere in either experiment, nor were there any significant

endophyte-associated shifts detected in overall rhizosphere community composition. Taxonomic analyses found that in both experiments endophyte infected plants had decreased abundances of a single abundant OTU compared with uninfected plants, which was found to be significant across both experiments (P= 0.026). The OTU sequence mapped with moderate (76-90%) homology to a number of reference sequences assigned as belonging to the class Sordariomycetes. Given previously observed endophyte-associated effects on arbuscular mycorrhizal (AM) fungi, reads assigned as belonging to AM were filtered and analysed separately. This showed that there were no significant effects of endophyte infection towards AM diversity nor overall community composition in both experiments, although there was an endophyte-associated increase in the abundance of the AM family Acaulosporaceae in the first experiment but not the second.

Thus, aside from an endophyte-associated antagonism towards an abundant OTU in the rhizosphere likely of the class Sordariomycetes, *E. festucae* var. *lolii* had an otherwise minor impact on the prokaryotic and fungal rhizosphere microbiome of their perennial ryegrass hosts. The minor magnitude of endophyte-associated effects was further emphasized by analyses consistently showing that both prokaryotic and fungal rhizosphere community composition differed to a greater extent between plants of each experiment irrespective of endophyte infection than between plants of differing endophyte status within each experiment- at least in this cultivar-endophyte strain interaction under the conditions of this study.

Acknowledgements

First and foremost I would like to thank Dr Jan Schmid for providing me with the opportunity to work on this project- I couldn't have asked for a better introduction to research. I've appreciated your continual guidance and support, but also for pushing me to make decisions for myself and always being encouraging of my ideas. I would also like to thank my cosupervisors Dr Dragana Gagic and Dr Richard Johnson for their continual support.

From Massey, I like to thank all other members of Jan Schmid's lab for being great to work around, especially Dr Ningxin Zhang and Asad for helping me overcome the molecular issues I had. Thanks to Olaf Greiwaldt for building the plant holders and associated equipment. Thanks to the Massey Genome Service/NZGL for their advice on sequencing and bioinformatics-related aspects of the project, particularly Dr Dave Wheeler and Associate Professor Patrick Biggs. Thanks also to Mauro Truglio for carrying out preliminary treatment and demultiplexing of the sequence data, and to Associate Professor Geoff Jones for carrying out the regression analysis.

From AgResearch, I'd like to thank Kim Richardson for supplying the plant material used for the project, Dr Jim Crush (AgResearch Ruakura) for advice regarding what plant growth conditions to use, and Anouck de Bonth for helping with the plantlet inoculation and for processing immunoblot sheets.

I am very grateful to the C. Alma Baker and Joan Dingley Memorial trusts for their financial support in 2016.

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Abbreviations

µL	Microliter(s)
16S	Prokaryotic small subunit ribosomal RNA gene
3D	Three-dimensional
AM	Arbuscular mycorrhiza
BLAST	Basic local alignment search tool
bp	Base-pairs
BS	Bulk soil
Bt	*Bacillus thuringiensis*
D	Simpson's diversity index
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E	Simpson's evenness index
E+	Endophyte-infected
E-	Endophyte-uninfected
E1	Experiment one
E2	Experiment two
EMP	Earth Microbiome Project
FC	Field capacity
FDR	False discovery rate
g	Gram(s)
h	Hour(s)
H_2O	Water
HCl	Hydrochloric Acid
ISR	Induced systemic resistance
ITS	Internal transcribed spacer region of the eukaryotic ribosomal RNA gene cluster
mg	Milligram
MHB	Mycorrhiza helper bacteria
MS	Murashige and Skoog medium

ng	Nanogram
OTU	Operational taxonomic unit
P	Phosphorus
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PERMANOVA	Permutational multivariate analysis of variance
PF	Plant-free
ppm	Parts per million
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative PCR
R	Rhizosphere
RO	Reverse osmosis
rpm	Revolutions per minute
rRNA	Ribosomal RNA
spp.	Species
TBE	Tris-borate-EDTA
UNITE	User-Friendly Nordic ITS Ectomycorrhiza Database
VOCs	Volatile organic compounds
x g	G-force

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1 | Introduction

1.1 The plant microbiome: A multi-compartmented 'second genome'

Soil is home to an enormous array of microbial life, with each gram typically containing millions of cells comprising thousands of microbial species (Torsvik & Øvreås, 2002). In particular the few millimetres of soil immediately surrounding plant roots hosts a far higher microbial density than the wider bulk soil, housing up to 10^{11} microbial cells per gram of plant root (Philippot et al., 2013). This zone is referred to as the rhizosphere, and its formation is the result of compounds secreted from plant roots referred to as root exudates, which creates a nutrient-rich zone in the few millimetres of soil surrounding the root surface (Bais et al., 2006). The rhizosphere is part of a wider multi-compartmented root microbiome that also collectively encompasses the rhizoplane (community living attached to the roots), and the endosphere (community living inside root tissue) (Gaiero et al., 2013) (Figure 1). In addition to the aboveground phyllosphere microbiome (Vorholt, 2012), the plant microbiome has been shown to play such an influence over the development and physiology of their hosts that it is regarded as the plants' 'second genome' (Berendsen, et al., 2012). Given its apparent malleability coupled with its importance, considerable interest exists to manipulate the microbiome to promote plant growth and physiology (Quiza & Yergeau, 2015; Zhang et al., 2015). The development and application of such strategies holds promise to give rise to novel sustainable approaches to agriculture, whereby inoculation of soils with bio-fertilizers among other microbial strategies should alleviate our often environmentally counter-productive overuse of pesticides and fertilizers (Lakshmanan et al., 2014; Sessitsch & Mitter, 2015). However, before the plant microbiome can be fully harnessed to better the quality of life of their hosts, a greater understanding of its structure and functions are still necessary (Berendsen et al., 2012).

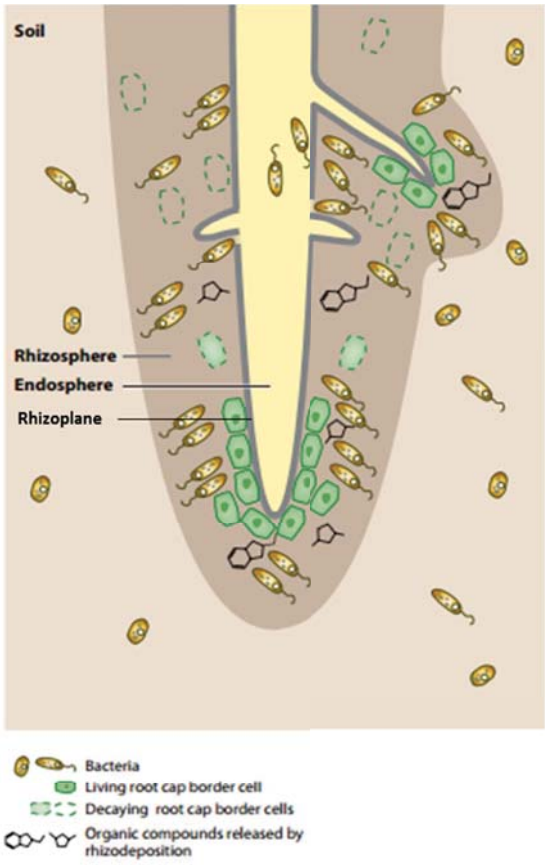

Figure 1. Niche differentiation at the root-soil interface. Illustration of the spatial compartmentalization of the rhizosphere (zone surrounding roots affected by root exudates (darker brown zone)), the rhizoplane (root surface) and the endosphere (inside root tissue). Figure adapted from Bulgarelli et al. (2013).

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Given that only a small proportion of microorganisms existing in nature can be cultured *in vitro*, traditional cultivation-dependent methods of microbial community analyses were severely limited in their ability to collectively analyse microbiomes as a whole (Lakshmanan et al., 2014). However, recent advances in next-generation sequencing technologies have made this possible by greatly enhancing the resolution with which complex microbial communities can be characterized (Segata et al., 2013). One commonly used method referred to as metabarcoding involves the high-throughput sequencing of polymerase chain reaction (PCR) amplicons of hypervariable regions of phylogenetic marker genes, allowing high-resolution taxonomic profiles of microbial communities to be generated (Taberlet et al., 2012). Alternatively, it is also possible to sequence whole metagenomic DNA (shotgun metagenomics) (Quince et al., 2017) or RNA (metatranscriptomics)

(Bashiardes et al., 2016), enabling additional insights into the functional capacity of microbial communities.

1.1.1 Determinants of rhizosphere community composition

Application of next-generation sequencing technologies in the context of the rhizosphere microbiome are greatly improving our understanding of the relative extent to which different factors influence rhizosphere community composition. The rhizosphere microbiome is dynamic in composition, both between different plants as well as throughout the life cycle of an individual plant (Aleklett & Hart, 2013). Aside from a relatively small number of microorganisms that are inherited from the seed surface or during seed dispersal, the vast majority of the root microbiome originates from the wider bulk soil in which the plant is growing (Philippot et al., 2013). Soil type therefore plays an overriding influence over rhizosphere community composition, as it determines the inventory of microorganisms present in the wider soil biome that can potentially colonize the rhizospheres of resident plants. However, rather than being passively colonized, plants also play a role in influencing community composition of their rhizospheres through the secretion of specific compounds from their roots. Additionally, anthropogenic factors such as the use of chemical pesticides and fertilizers among other agricultural practices such as widespread plant monoculture also impact rhizosphere community composition. This section describes these determinants of rhizosphere community composition in further detail.

1.1.1.1 Abiotic factors

Given that the rhizosphere microbiome is largely obtained from the wider bulk soil, the factors that influence composition of the soil biome inherently also play an overriding role in determining rhizosphere community composition. The composition of the soil biome is determined by a range of abiotic factors including pH (Lauber et al., 2009), temperature and wider climatic conditions (Ward et al., 1998), soil water content (Manzoni et al., 2011; Nessner Kavamura et al., 2013), salinity (Nubel et al., 2000), and nutrient status (Broughton

& Gross, 2000). Next-generation sequencing studies of a range of different soil types have demonstrated that soil pH has a particularly strong influence on the taxonomic composition of the bacterial soil biome (Lauber et al., 2009; Rousk et al., 2010). On the other hand, fungi appear less affected by pH (Rousk et al., 2010) and are instead more strongly influenced by seasonal variation and climatic factors (Dumbrell et al., 2011; Tedersoo et al., 2014).

1.1.1.2 Biotic factors

It is well established that plants also play an active role in determining the composition of their microbiomes (Hartmann et al., 2009), to such an extent that in some instances even minute changes in plant genotype can affect rhizosphere community composition (Aira et al., 2010; İnceoğlu et al., 2010; Lundberg et al., 2012). Plants can select for microbial communities by altering the nutrient composition of their rhizospheres through the root exudates they secrete (Bais et al., 2006; Hartmann et al., 2009), and even release metabolically active cells from the root cap (root border cells) into the rhizosphere (Hawes et al., 1998). In some instances, this selection involves the recruitment of beneficial microorganisms (Rudrappa et al., 2008), while in others root exudates can instead deter pathogens (Baetz & Martinoia, 2014; Bais et al., 2005) (Figure 2). The influence plants have on determining community composition of their rhizospheres was clearly shown in a study characterizing rhizosphere community composition and root exudation of a range of *Arabidopsis* ATP-binding cassette (ABC) (Rea, 2007) transporter mutants, which found that mutants with altered exudation profiles consequentially hosted different bacterial communities in their rhizosphere microbiomes (Badri et al., 2009). Furthermore, it was recently shown that different genotypes of *Arabidopsis* react differently to the stimulatory effects of a *Pseudomonas simiae* strain on lateral root formation, illustrating that in some instances even small genotypic variation within-species can affect the efficacy of beneficial rhizosphere microorganisms (Wintermans et al., 2016).

Despite the clear role that root exudation plays in determining rhizosphere community composition it has been argued that the role of root exudates in determining rhizosphere microbiome structure may be overrepresented. While the authors do not intend to suggest

that root exudates are unimportant to structuring the rhizosphere, Dennis et al (2010) suggest that root exudates may not necessarily be the predominant pool of rhizodeposits that influence rhizosphere community composition, as upon release from root apices root exudates are rapidly mineralized and re-released by microorganisms into the rhizosphere. This perspective places increased importance on microorganisms towards determining the resulting structure of the rhizosphere microbiome. However, the influence that plant-derived root exudates have on those microorganisms which initially colonize their rhizospheres could have flow-on effects on the development of the eventual established community, similarly to that seen in the human oral microbiome where primary colonizers influence subsequent colonization of other taxa (Rickard et al., 2003).

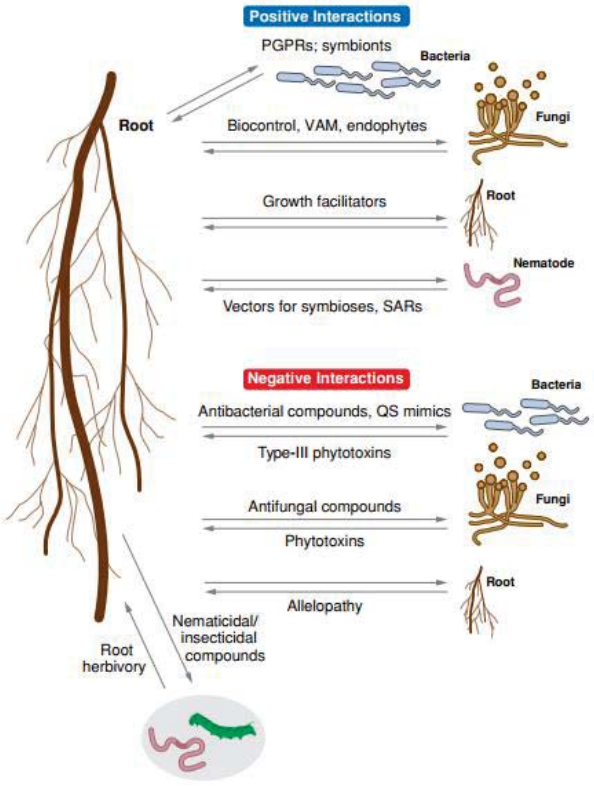


Figure 2 Schematic representation of possible rhizospheric interactions mediated by root exudates. Root-mediated rhizospheric interactions are broadly classified into two categories, positive and negative interactions. Positive interactions involve root exudate-mediated interactions with plant growth–promoting rhizobacteria (PGPR). Roots produce chemical signals that attract bacteria and induce chemotaxis. Positive interactions mediated by root exudates also include growth facilitators or growth regulator mimics that support growth of other plants and also perform cross-species signalling with rhizospheric invertebrates. In contrast, negative interactions mediated by root exudates involve secretion of antimicrobials, phytotoxins, nematicidal, and insecticidal compounds. The arrows in the panels indicate chemical exchange. VAM, vesicular arbuscular mycorrhizas; SARs, systemic acquired resistance. Figure and legend adopted from Bais et al. (2006).

1.1.1.3 Anthropogenic factors

Modern agriculture involves the widespread use of artificial fertilizers, which provides short-term relief of nutrient stress to crops (Tilman et al., 2002). However, these intensive farming practices have also been shown to impact rhizosphere community composition of plants growing in fertilized soils (Figure 3). A study assessing the rhizosphere and root exudate profiles of maize (*Zea mays*) plants found that high levels of nitrogen fertilization caused plants to secrete larger amounts of sugars and phenolics (Zhu et al., 2016), and has been shown to affect fungal community composition of the rhizosphere of sugarcane plants (Paungfoo-Lonhienne et al., 2015). High phosphorus levels have also been shown to inhibit the growth of some arbuscular mycorrhizal (AM) fungi (Breuillin et al., 2010; Menge et al., 1978), as well as phosphate-solubilising Actinobacteria (Mander et al., 2012; Wakelin et al., 2012). Thus, the abundance of nutrients supplied to plants through external chemical application may alleviate the need for plants to expend energy recruiting and accommodating beneficial rhizosphere microorganisms to thrive in their environments.

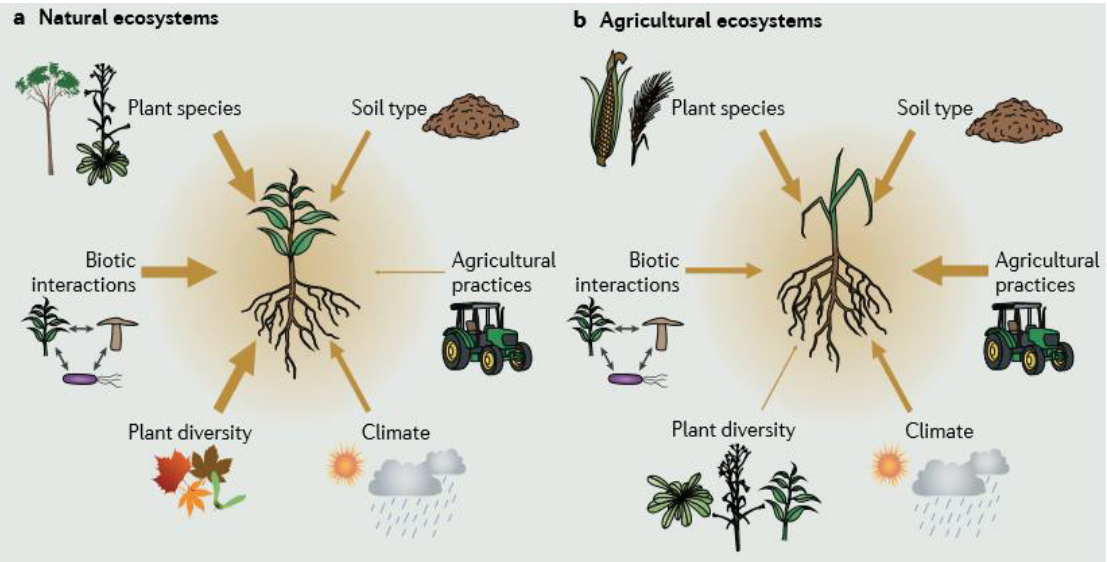


Figure 3. Differences in factors impacting rhizosphere community composition in natural versus agricultural settings. Illustration of the relative impacts of different factors on the rhizosphere microbiome in **a)** natural systems, compared with **b)** agricultural ecosystems. The relative importance of each factor towards rhizosphere community composition in each context is represented by the thickness of the arrow. Figure adopted from Phillipot et al. (2013) with permission from Macmillan Publishers Ltd through RightsLink.

Another common characteristic of modern agriculture is the widespread monoculture of agronomically relevant crops, which heavily contrasts the highly diverse communities that plants typically exist within in nature (Philippot et al., 2013). Plant-soil feedbacks refer to the reciprocal effects that aboveground and belowground biomes have on one another, and occur through a wide range of different mechanisms with plants acting as a 'bridge' between these two spatially separated biomes (Wardle et al., 2004). As plants are the primary producers of nutrients for the soil biome, its composition is heavily influenced by the plant communities living in the soil. This has been shown in studies that have compared soil microbiome composition of soils from natural settings of high plant diversity and single agricultural monocultures (Bakker et al., 2013), as well as experiments that have assessed soil biomes in plots consisting of a gradient of plant species richness (Bakker et al., 2014). For example, the antagonistic activity of *Streptomyces* spp. towards pathogenic bacteria tends to decrease as plant community diversity increases, suggesting that in diverse settings plants do not rely as heavily on this antagonistic activity of *Streptomyces* than under lower plant diversity (Bakker et al., 2013). At the same time however, plant monoculture has also been shown to be associated with the development of disease-suppressive soils (soils which resist pathogen invasion under conditions which would normally allow pathogenicity (Kinkel et al., 2011)). In either instance, the results of these studies illustrate the impact that aboveground plant diversity has on belowground soil microbial diversity.

1.1.2 Effects of the root microbiome on their hosts

The specific consortia of microbes comprising the root microbiome (collective rhizosphere, rhizoplane and endosphere) of a given plant has wide-reaching effects on the development and physiology of their hosts (Berendsen et al., 2012). Microbial strategies of promoting plant growth include the production and secretion of beneficial plant hormones (phytostimulation), antagonism towards pathogenic microorganisms (biocontrol), and aiding the plant in nutrient acquisition (biofertilization) (Gaiero et al., 2013). This section discusses each of these mechanisms in further detail, before outlining the importance of both the rare biosphere and the collective structure of the root microbiome as a whole.

1.1.2.1 Microbial mechanisms of plant growth promotion

Phytostimulation is defined as the direct promotion of plant growth through the production and secretion of plant hormones (Bloemberg & Lugtenberg, 2001), and is a widely prevalent mechanism employed by a range of root-inhabiting microorganisms to enhance the growth of their hosts (Gaiero et al., 2013). Ethylene is a plant hormone essential for normal plant growth and development, and its concentration is regulated by an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick, 2014). However, an accumulation of ACC deaminase negatively regulates ethylene levels as it is responsible for cleaving ACC, which is a precursor for ethylene biosynthesis (Glick, 2014). ACC deaminase is synthesized by a number of rhizosphere microbes and therefore the bacterial presence and secretion of ACC deaminase can lower plant ethylene levels, in turn increasing plant growth as well as providing increased salt tolerance and drought resistance to their hosts (Ahemad & Kibret, 2014). A study screened 20 ACC deaminase producing rhizobacteria for their effectiveness at promoting growth in maize (*Zea mays*) over a gradient of increasing salinity, which identified six bacterial strains that significantly promoted a range of plant growth parameters including root and shoot length. Interestingly, the plant growth promoting effects were most pronounced at the highest level of salinity that was tested (Nadeem et al., 2007). It has been suggested that the widespread application of ACC deaminase producing bacteria could represent a paradigm shift in agriculture, making it easier to feed the growing global population in decades to come (Glick, 2014).

Certain root-associated microbes act as biofertilizers by facilitating the uptake of nutrients by their hosts (Bhardwaj et al., 2014). One of the most widely studied examples of biofertilization occurs in symbioses between >90% of land plants and arbuscular mycorrhizal (AM) fungi (Parniske, 2008). The interaction involves an initial chemical-chemical signalling between plant and fungus, which eventually leads to a more intimate molecular crosstalk that instigates the hyphal invasion and formation of structures (arbuscules) inside root cortical cells (Oldroyd, 2013). In this mutualism, the fungus scavenges nutrients from the soil and provides it to the plant in exchange for a source of nutrients that it solely relies on the plant for. Despite their promise as bioinoculants however, their inability to be cultured in the absence of their hosts as well as their high

degree of host specificity have so far impeded their widespread implementation (Berruti et al., 2016). While biofertilization is the main benefit AM are associated with, in some contexts certain AM species can also protect their hosts from infection from pathogens (Wehner et al., 2010), and have even been recently shown to facilitate phytoremediation (Pilon-Smits, 2005) of their hosts (Yang et al., 2016).

As opposed to being directly antagonistic towards pathogens (Whipps, 2001), some rhizosphere microorganisms can instead partake in molecular dialogue with the plant that confers a heightened state of immunity, thereby 'priming' (Conrath, 2011) their hosts to resist pathogen invasion (Pieterse et al., 2014). The process of this heightened immune state induced by beneficial microbes is referred to as induced systemic resistance (ISR), and can be an extremely effective means of combatting plant disease (Pieterse et al., 2014). Since its discovery in the early 1990s (Alstrom, 1991; van Peer et al., 1991; Wei et al.,1991), an extensive body of research has focused on better understanding the molecular mechanisms underlying the response. An experiment examining the effect of treatment with a root-associating *Pseudomonas putida* strain found that this triggered induced systemic resistance in the model plant *Arabidopsis thaliana* from Fusarium wilt disease. The experiment further illustrated that ISR is dependent on the plant hormones ethylene and jasmonic acid, as well as the transcriptional co-regulator NPR1 (Ahn et al., 2007). Extensive research has been undertaken to elucidate the complete genetic pathway underlying ISR, which has so far revealed that the transcription factor MYB72 is an early root-specific regulator essential for ISR (Van der Ent et al., 2008). Furthermore, it was recently shown that an enzyme called BGLU42 acts downstream of MYB72 in the roots of *Arabidopsis*, and that both MYB72 and BGLU42 are also involved in the regulation of iron uptake (Zamioudis et al., 2014). Continuing to unravel the molecular mechanisms underlying ISR should facilitate the development of strategies in the future enabling its induction.

1.1.2.2 The importance of the rare biosphere

While microbial communities often consist of enormous numbers of different species, the vast majority of which are often present in very low relative abundances, where only a

small fraction of this diversity comprises the majority of the overall biomass of the community (Nemergut et al., 2011). However, despite its low overall relative abundance the rare biosphere is considered a hidden driver of microbiome function, as a number of rare species have been shown to play important roles in microbiomes (Jousset et al., 2017). For example, dilution-to-extinction experiments have shown that bacterial species present in low abundances are involved in the production of antifungal volatile organic compounds (VOCs) that protect against soil-borne fungal pathogens (Hol et al., 2015). Similarly, it was shown that a species with a 16S ribosomal RNA (rRNA) relative abundance of 0.006% plays an important role in sulfate reduction in a natural peatland (Pester et al., 2010). The sequencing depth required to adequately characterize the rare biosphere as well as the difficulty differentiating sequence artefacts from genuine rare species remain challenges (Zhan & MacIsaac, 2015), however the continual increases in accuracy and decreases in costs of next-generation sequencing technologies (Goodwin et al., 2016) will inevitably facilitate future assessments of the roles of the rare biosphere in microbiome functioning.

1.1.2.3 The importance of the microbiome as a whole

While individual members of a given microbiome may influence plant health through the abovementioned mechanisms, the efficacy with which they are able to do so is largely reliant on the structure of the microbiome as a whole. Interactions within the context of plants growing in soil are extensive- with plant-plant, plant-microbe, and microbe-microbe interactions together collectively shaping the composition of the rhizosphere microbiome (Bakker et al., 2014) (Figure 4). Many of the species residing in the rhizosphere are commensals that do not directly interact with their host plants, but instead indirectly do so by interacting with plant growth promoting species through microbe-microbe interactions, or by being antagonistic towards pathogenic microbes. For example, mycorrhiza helper bacteria (MHB) benefit their plant hosts by being synergistic towards AM (Schrey et al., 2005), thereby indirectly facilitating the direct benefits AM provide to their plant hosts. Similarly, *Streptomyces* indirectly facilitate the plant by being antagonistic towards pathogenic microbes (Bakker et al., 2013)- although *Streptomyces* are also known to impart some of their benefits through directly interacting with their hosts as well (Tokala

et al., 2002). This emphasizes the benefits of next-generation sequencing-based approaches to characterize complex microbial communities, as these technologies possess the throughput capable of generating the vast amounts of data required to collectively encapsulate the magnitude of diversity often present in microbiomes.

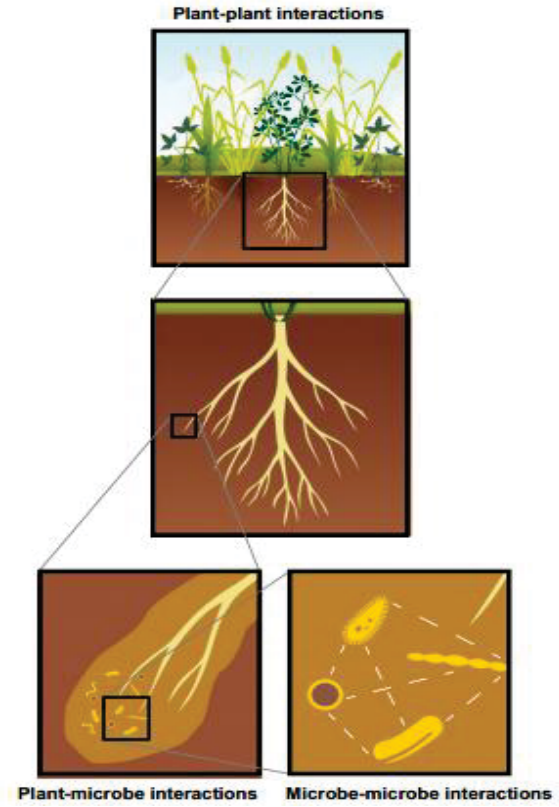


Figure 4. Species interactions at diverse scales can influence soil biome composition, structure and functioning. Most attention to date has been given to direct plant-microbe interactions, taking place in an environment under plant influence, are vital in shaping microbiome structure and functioning. Furthermore, plant-plant interactions can alter host plant impacts on associated soil microbiomes. Figure and legend adopted from Bakker et al (2014) with permission through RightsLink.

1.2 *Epichloë*-Grass symbiosis

One particularly well-studied member often present in the endophytic microbiome of a range of temperate grasses of the subfamily pooideae are *Epichloë* endophytes, which inhabit the apoplastic spaces of aboveground parts their hosts (Tanaka et al., 2012). Endophytic growth in the host typically involves single seldom branching hyphae in the

apoplastic spaces between plant cells (Figure 5), where the endophyte grows by intercalary extension synchronous with the growth of their hosts (Christensen et al., 2008). The endophyte is provided with its sole source of nutrients from their plant hosts, while in return their hosts are often provided heightened resistance from biotic and abiotic stresses. However, each symbiosis exists on a mutualistic-parasitic continuum (Müller & Krauss, 2005) where each endophyte strain/plant cultivar interaction results in unique biological outcomes (Johnson et al, 2007), which are also further influenced by the wider environmental conditions (Hesse et al., 2003). Little is currently known of the molecular mechanisms underlying this variability in biological outcomes of different host-endophyte combinations.

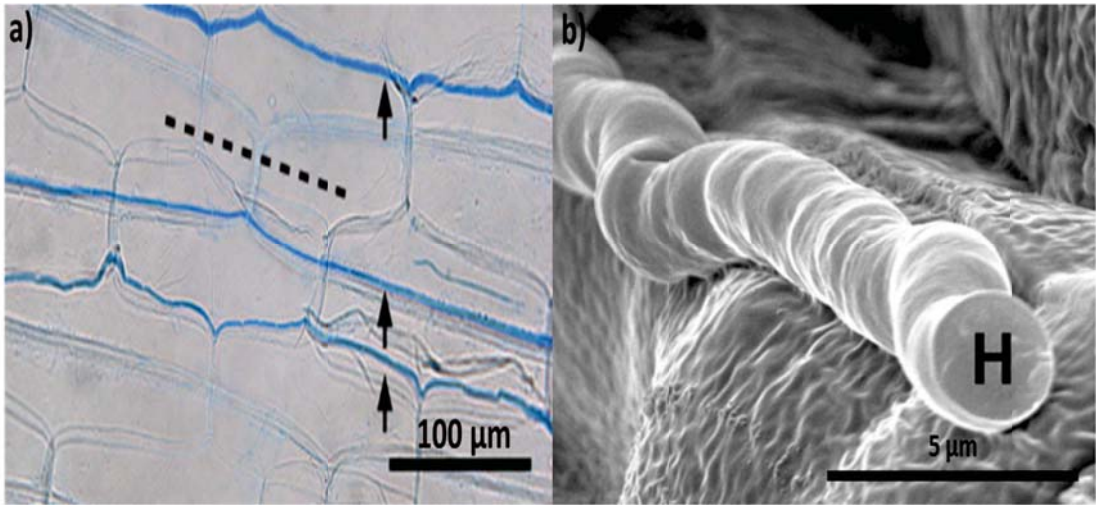


Figure 5. Growth of *Epichloë* endophytes within their grass hosts. **a)** *E. festucae* var.*lolii* in a ryegrass leaf-sheath stained with aniline blue (arrows), are oriented parallel to the longitudinal leaf axis (dashed line). b) Freeze-fracture scanning electron micrograph (SEM) image showing a hypha (H) of *E. coenophiala* in close contact with tall-fescue leaf cells. Figure and legend adapted from Christensen et al (2008) with permission from RightsLink.

1.2.1 Effects of *Epichloë* endophytes on their hosts

The most well-understood effects of *Epichloë* endophytes on their hosts are due to their production of bioactive alkaloids exclusively when growing *in planta*, which provides protection to their hosts from insect herbivory (Schardl et al., 2012). Biosynthesis of peramine (Rowan & Gaynor, 1986), lolines (Schardl et al., 2007) and epoxy-janthitrems

(Popay & Wyatt, 1995) are associated with these insect-deterrent effects. However, some strains also secrete alkaloids that cause costly toxicosis of grazing livestock, such as ergovaline (Yates et al., 1985) and lolitrem B (Gallagher et al., 1981). Consequentially, endophyte strains have been developed and commercialized which produce agriculturally desirable alkaloids providing resistance to crops from insect herbivory, without synthesizing those deleterious to the health of grazing livestock (Johnson et al., 2013). In addition to these few well-studied alkaloids however, a variety of other metabolites have been identified as being present at different levels in different symbioses (Rasmussen et al., 2008a). This suggests the existence of additional currently uncharacterized metabolites that may potentially play important roles in determining the biological outcomes of a given plant genotype-endophyte strain interaction, which may additionally have implications for insect herbivores (Rasmussen et al., 2008b).

Abiotic stress resistance imparted by *Epichloë* endophytes appears a more variable phenomenon in *E. festucae var. lolii*-infected perennial ryegrass than in *E. coenophiala*-infected tall-fescue (West, 1994). While some studies have shown positive effects (Hahn et al., 2008; Ravel et al., 1997), others have instead shown either no effect (Barker et al., 1997) or even negative effects (Eerens et al., 1998) of endophyte infection on host resistance to drought. It was recently shown in perennial ryegrass infected with *E. festucae* (Fl1) that endophyte infection reduced the expression of heat-responsive drought-related genes in infected plants compared with uninfected plants, suggesting that rather than enhancing a response to drought by upregulating drought-responsive genes, endophyte infection instead decreases host sensitivity to drought (Dupont et al., 2015). On the other hand, in the symbiosis between *E. festucae* var. *lolii* (Lp19) and perennial ryegrass, endophyte infection instead caused an upregulation of genes involved in protection of the plant from abiotic stresses such as genes under the control of WRKY transcription factors (Phukan et al., 2016). Upregulation of WRKY-controlled genes was also recently shown to be associated with endophyte infection in *E. coenophiala*- infected tall-fescue (Dinkins et al., 2017). These differences in impacts on host gene expression in different symbioses suggest there may be multiple strategies by which different *Epichloë* endophytes can impart abiotic stress resistance to their hosts in certain contexts, although it is still unclear why these effects are observed only in some studies but not others.

1.2.2 Novel insights through comparative omics'

Omics' techniques involve the parallel measurement of all genes (genomics), mRNA (transcriptomics), proteins (proteomics) or metabolites (metabolomics) of a given sample (Horgan & Kenny, 2011), and have enabled ground breaking insights into plant-microbe interactions (Knief, 2014). In the context of *Epichloë*-grass symbiosis, a wide range of comparative transcriptomics, metabolomics and proteomics studies comparing endophyte infected plants with uninfected plants have greatly improved our understanding of the molecular basis underlying effects that *Epichloë* endophytes have on their hosts (Johnson et al., 2007; Tanaka et al., 2012). A study assessing the transcriptome of perennial ryegrass infected or uninfected with *Epichloë festucae* (Fl1) found that endophyte infection alters the gene expression of over one third of host genes - far more so than other well studied symbioses typically do, and in this sense more closely resembles a pathogenic interaction (De Cremer et al., 2013; Doehlemann et al., 2008; Kawahara et al., 2012). The study found endophyte infection to globally reduce primary metabolism of their hosts, while upregulating genes involved in secondary metabolism (Dupont et al., 2015). In contrast, another recent transcriptomics study assessed the native symbiosis between perennial ryegrass (NuiD) infected with *Epichloë festucae* var. *lolii*, and found endophyte infection caused a systemic upregulation of genes rather than the downregulation observed by Dupont et al (2015), with little overlap in the genes that were differentially expressed (Schmid et al., 2017).

It is well known that gene expression levels correlate poorly with protein levels (Maier, et al., 2009), thus emphasizing the importance of complementing investigations of the transcriptome with proteomics and metabolomics studies. A comparison of the proteomes of perennial ryegrass plants infected and uninfected with *E. festucae* var. *lolii* (Lp19) revealed that a fungal superoxide dismutase protein was expressed at detectable levels in extracts collected from infected plants, but not uninfected plants. Higher levels of pathogenesis-related 10 (PR-10) protein (van Loon et al., 2006) were also detectable in plants infected with an endophyte displaying restrictive growth *in planta* (characteristic of a mutualistic interaction), while not in plants infected with an endophyte strain that typically exhibits unrestrictive proliferative growth *in planta*. This suggests that the elicitation of a mild host immune response may play a role in maintaining restrictive hyphal

growth (Zhang et al., 2011). Comparison of the metabolome of perennial ryegrass plants infected with three different endophyte strains or uninfected revealed that endophyte infection alters a far wider range of host metabolites than those few well-studied fungal alkaloids (Rasmussen, et al., 2009). Integration of data generated in these analyses as well as those of future omics' studies will continually contribute towards a systems biological understanding of the molecular framework underlying *Epichloë*-grass symbiosis.

1.2.3 *Epichloë*-induced impacts towards the root microbiome

Despite being virtually completely absent from roots (Christensen & Voisey, 2007), *Epichloë* endophyte infection has been shown to impact the biogeochemical conditions and microbial community composition within the rhizosphere of their hosts. Field studies have found that pastures dominated by endophyte-infected tall-fescue contain higher total organic carbon and nitrogen than pastures with low endophyte frequency (Franzleubbers & Nazih, 1999; Franzluebbers, 2006; Iqbal et al., 2012). Infection of tall-fescue with *E. coenophiala* alters root morphology (Malinowski et al., 1999), increases the concentration of phenolics in roots, and also facilitates the ability of roots to accumulate phosphorus under phosphorus-deficient conditions (Malinowski et al., 1998). Endophyte-infected tall-fescue has also been shown to have greater biomass when grown in soil previously inhabited by endophyte-negative than endophyte-positive plants, suggestive of an endophyte-associated soil-mediated negative-feedback mechanism (Matthews & Clay, 2001). Furthermore, treatment of soil with rhizodeposits obtained from *E. coenophiala*-infected tall-fescue plants has been shown to stimulate soil microbial activity to a greater degree than rhizodeposits obtained from uninfected plants (Van Hecke et al., 2005). Similarly, a recent study assessed aseptically growing tall-fescue plants infected with different *E. coenophiala* strains and found strain-specific differences in the composition of root exudates secreted from plant roots (Guo et al., 2015). Fewer studies have so far assessed these effects in perennial ryegrass, however infection of perennial ryegrass with the commercialized AR1 (Fletcher, 1999) and AR37 (Popay & Wyatt, 1995) endophyte strains has been shown to induce shifts in various n-alkane hydrocarbon compounds in the rhizosphere metabolome of their hosts (Wakelin et al., 2015).

1.2.3.1 Effects of endophyte infection towards arbuscular mycorrhizal fungi

Despite their spatial separation within their grass hosts (Figure 6), numerous studies have shown interactions between foliar *Epichloë* endophytes and root-dwelling arbuscular mycorrhizal (AM) fungi. Studies specifically measuring endophyte and AM concentrations have illustrated antagonism between *Epichloë* endophytes and certain AM species, such as *Sclerocystis spp.* (Muller, 2003) and *Glomus spp.* (Liu et al., 2011). A study by Guo et al (1992) found that *E. coenophiala* infection of tall-fescue did not necessarily affect infection of *Glomus mosseae*, however it significantly reduced subsequent mycorrhizal colonization and reproduction in their hosts. The authors posit this as potentially being evidence of the role of bioactive alkaloids, as newly infected seedlings would not yet have a high enough concentration for any effect to be observed. Given both fungi obtain carbon from their hosts and that *Epichloë* are closer to the site of photosynthesis in the leaf, it was also hypothesized that *Epichloë* may have 'first priority' and therefore out-compete AM for nutrients (Guo et al., 1992). On the other hand however, in the wild grass *Bromus auleticus*, infection with *Epichloë pampeana* instead increases the diversity of AM fungi (Arrieta et al., 2015; Vignale et al., 2015). A recent study in tall-fescue infected *E. coenophiala* found that endophyte infection had no impact on belowground AM communities (Slaughter & McCulley, 2016), showing that endophyte infection does not always affect AM community composition of their hosts. Given the role AM fungi play in structuring plant communities (Lin et al., 2015; Marcel et al., 1998), *Epichloë*-induced impacts towards AM fungi could also have important implications for the structure of the wider plant community in which they are growing.

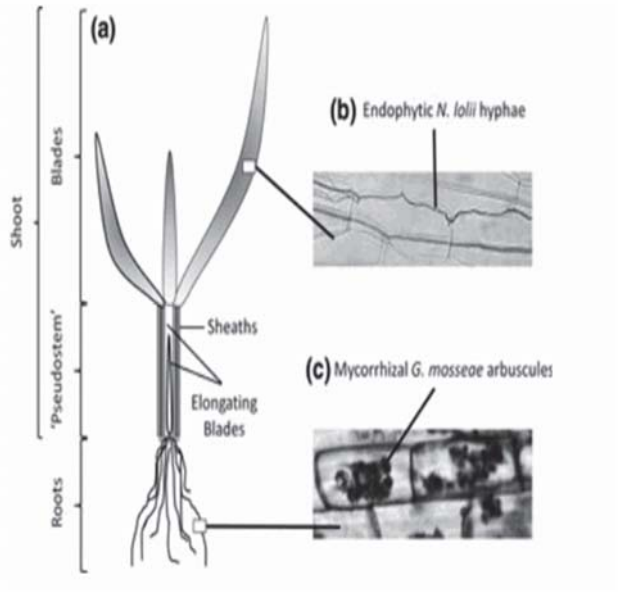


Figure 6. Spatial separation of *Epichloë* endophytes and AM fungi within their hosts. Schematic diagram of the anatomy of a dually infected grass plant **a)** showing endophytic *E. festucae var. lolii* hyphae in leaf blades **b)** and colonisation by mycorrhizal arbuscules in root tissues **c)**. Figure and legend adopted from Liu et al (2011) with permission obtained through RightsLink.

1.2.3.2 Cultivation-independent studies

Despite the insights reached through the targeted analyses of well-characterized microbial groups, given that the vast majority of microorganisms existing in nature are unculturable (Rappe & Giovannoni, 2003) requires the use of cultivation-independent techniques to globally assess root-associated microbial communities. Studies using denaturing gradient gel electrophoresis (DGGE) have shown that *Epichloë* infection alters the bulk soil biome of Italian ryegrass (Casas et al., 2011) and perennial ryegrass (Bell et al., 2009). A recent analysis of perennial ryegrass infected with two widely used commercial *E. festucae* var. *lolii* strains (AR1 and AR37) using DGGE showed that endophyte infection affects both bacterial and fungal rhizosphere community structure, however specific targeting of *Pseudomonas* revealed that this group of bacteria were not affected by endophyte infection (Wakelin et al., 2015). However, while fingerprinting techniques such as DGGE provide a broader assessment of microbial diversity than cultivation-dependent approaches, they are unfortunately incapable of readily depicting the collective taxonomic composition of microbiomes- nor can they provide any insights into their functional capacity (Chaparro et al., 2014).

1.2.3.3 High-throughput studies

High-throughput sequencing has revolutionized microbial ecology by greatly enhancing the resolution with which complex microbial communities can be characterized (Cardenas & Tiedje, 2008), although studies implementing these technologies in the context of *Epichloë*-grass symbiosis have so far remained scarce. Furthermore, to date all such studies have been carried out in the closely related yet distinct symbiosis between tall-fescue (*Festuca arundinacea*) and *Epichloë coenophiala*. Metabarcoding of the bacterial rhizosphere community of tall fescue in response *to E. coenophiala* infection revealed that, similarly to phyllosphere communities (Roberts & Lindow, 2014), endophyte infection exerts selection pressure on loline-catabolizing bacteria in the rhizosphere, as loline secreted by the fungus is translocated to the roots of their hosts (Kimmons, 1990). Interestingly, the study also found that inoculation of endophyte-infected rhizospheres with a loline-catabolizing *Burkolderia ambifaria* strain led to a shift in rhizosphere community composition, with Firmicutes present in a higher relative abundance than in the rhizospheres of plants instead inoculated with a non loline-catabolizing strain (Roberts & Ferraro, 2015). Another recent study assessed the impact of *E. coenophiala* infection towards rhizosphere community composition of tall-fescue plants growing under field conditions, and found endophyte-associated effects towards the fungal rhizosphere community, with subtle but significant increases in Glomeromycota and decreases in Ascomycota in the rhizosphere of infected versus uninfected plants. On the other hand, the prokaryotic rhizosphere microbiome was not significantly affected by endophyte infection in this study (Rojas et al., 2016).

1.3 Project outline

Given the results of a recent DGGE analysis of the rhizosphere microbiome of perennial ryegrass showing that inducing shifts in both bacterial and fungal community composition (Wakelin et al., 2015) coupled with the recent results of a transcriptomics study which found endophyte induced alterations in the virtually completely uncolonized root of their hosts in this particular cultivar-strain interaction (Schmid et al., 2017), the objective of this study is to assess prokaryotic and fungal rhizosphere community composition of perennial

ryegrass (NuiD) in response to *E. festucae* var. *lolii* (Lp19) infection using high-throughput metabarcoding. To control for effects that can arise due to genotypic effects, all plant material used in this study originated from clonal plantlets growing in sterile tissue culture, thereby also meaning that replicates were genotypically identical and did not have any previously inherited microbes living in association with them before their use in each experiment. To assess the reproducibility of any endophyte-associated effects, two independent experiments were carried out using soil collected from a natural ryegrass pasture at two separate times of the year. After approximately two months of growth under controlled conditions in a growth cabinet, rhizospheres of plants were sampled and prokaryotic and fungal amplicons were sequenced and processed using established bioinformatic tools, enabling the comparison of both levels of diversity and taxonomic composition within the rhizosphere microbiome of each plant. While the bacterial rhizosphere of perennial ryegrass has been previously assessed using metabarcoding (Chen et al., 2016; Lagos et al., 2014), to our knowledge this is the first study to assess the impact of *E. festucae* var. *lolii* infection on the rhizosphere microbiome of perennial ryegrass using next-generation sequencing technologies.

2 | Materials and Methods

2.1 Materials

2.1.1 Media

All media was sterilized for 15 minutes at 121^{o}C prior to use, and was prepared using milli-Q H_2O. When antibiotics were added, media was cooled to approximately 50^{o}C prior to being added. Any media that was not immediately used was stored at 4^{o}C prior to use. Where necessary, the pH of solutions was adjusted with a pH meter (Model PHM210, Radiometer Copenhagen) with a HI1230B electrode (Henna instruments).

Potato Dextrose Agar (PDA)

PDA contained 24 g/L of dehydrated potato dextrose broth (Difco), and was prepared by addition of 15 g/L of agar (Acumedia).

Water agar (WA)

Water agar was prepared to 1.5% using 15 g/L agar (Acumedia).

Murashige and Skoog (MS) media

MS (Murashige & Skoog, 1962) media was prepared to 1 litre with the following: 30 g sucrose, 4.4 g Murashige and Skoog salts (Sigma-Aldrich), 1 ml of nutrient solution (0.3 g/L cytokinin, 0.2 g/L iron, 0.002 g/L Thiamine and 0.05 g/L Inositol) and 15 g agar (Acumedia). Prior to autoclaving, pH was adjusted to 5.7 using NaOH.

2.1.2 Buffers

Phosphate Buffered Saline (PBS)

1X PBS was prepared to 1 litre following the Cold Spring Harbor protocol ("Phosphate-buffered saline (PBS)", 2006), using 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 8 g NaCl, and 0.2 g KCl (final pH= 7.4).

Tris-Borate-EDTA (TBE)

TBE was made to 10X following the Cold Spring Harbor protocol ("TBE buffer", 2006), and was prepared to 1 litre using 121.1 g tris base, 61.8 g boric acid, and 7.4 g EDTA.

Tris-HCl

All Tris-HCl used was either elution buffer provided with DNA Powersoil DNA extraction kits (Solution C6), or elution buffer provided with Roche High-Pure PCR purification kits (10 mM, pH 8-8.5).

2.2 Methods

2.2.1 Plantlet propagation and inoculation

Clonal *Lolium perenne* 'NuiD' (Tan et al., 1997) plantlets growing in sterile tissue culture were obtained from Kim Richardson (AgResearch Ltd., New Zealand), and were further propagated in MS media by aseptically excising tufts of tillers and individually placing them in fresh media. *Epichloë festucae* var. *lolii* (Lp19) was isolated from plants which had been successfully inoculated with Lp19 12 years prior by aseptically plating surface-sterilized (washed in 96% ethanol for a few seconds followed by 10% Bleach ('Janola Premium Bleach' (<5% sodium hypochlorite)) for 2-4 minutes followed with three rinses with sterile water) pseudostems on PDA and incubating plates at 22^{o}C in the dark. Endophyte-infected plantlets were generated using the method described in Latch & Christensen (1985), whereby a small piece of endophyte was aseptically inserted into a slit made in the meristematic region of plantlets on water agar. Plantlets were screened for infection using the immunoblotting procedure outlined in Simpson et al (2012). A tiller from each plant was cut near the base with scissors, and the newly cut surface of the tiller was then pressed onto nitrocellulose paper before being sent for processing at AgResearch Grasslands Research Centre (Palmerston North, New Zealand). Plantlets deemed successfully inoculated by immunoblots turning a red/pink colour following processing (Appendix A1) were screened a second time to further confirm their infection status, and were thereafter propagated separately to uninoculated material by aseptically separating clusters of tillers and incubating them in a growth cabinet at 22^{o}C set to a 12/12h day/night cycle in fresh MS media.

2.2.1.1 Screening inoculated plantlets for endophyte infection

Due to residual endophyte growing into the agar surrounding the base of inoculated tillers (Figure 7 b) which could have triggered false-positive blot readings, this problem was circumvented using the screening procedure outlined in Figure 7 c. Each inoculated tiller was firstly grown in MS media until a cluster of 3-5 tillers was formed. Next, from each cluster a single tiller was aseptically excised and placed in fresh MS media and assigned a number. The remainder of the cluster was then placed in soil in 40 cm^3 plastic root trainers and allowed to grow for 2-3 weeks under experimental conditions. Following this, plants growing in soil were tested for endophyte infection using the endophyte-specific immunoblot assay described in Simpson et al (2012). Of the 39 plants tested, number 25 gave a particularly strong positive signal (Appendix A1) and was therefore tested further by re-blotting additional propagated tillers originating from number 25 alongside two other potentially inoculated tillers, as well as by plating surface-sterilized pieces of pseudostem on Potato Dextrose agar (PDA) and incubating them at 22^oC in the dark. This confirmed number 25 as being successfully inoculated with endophyte (Figure 7 a, Appendix A1), and therefore tillers deriving from number 25 were further propagated separately in MS media and thereafter used as endophyte-positive material in plant growth experiments.

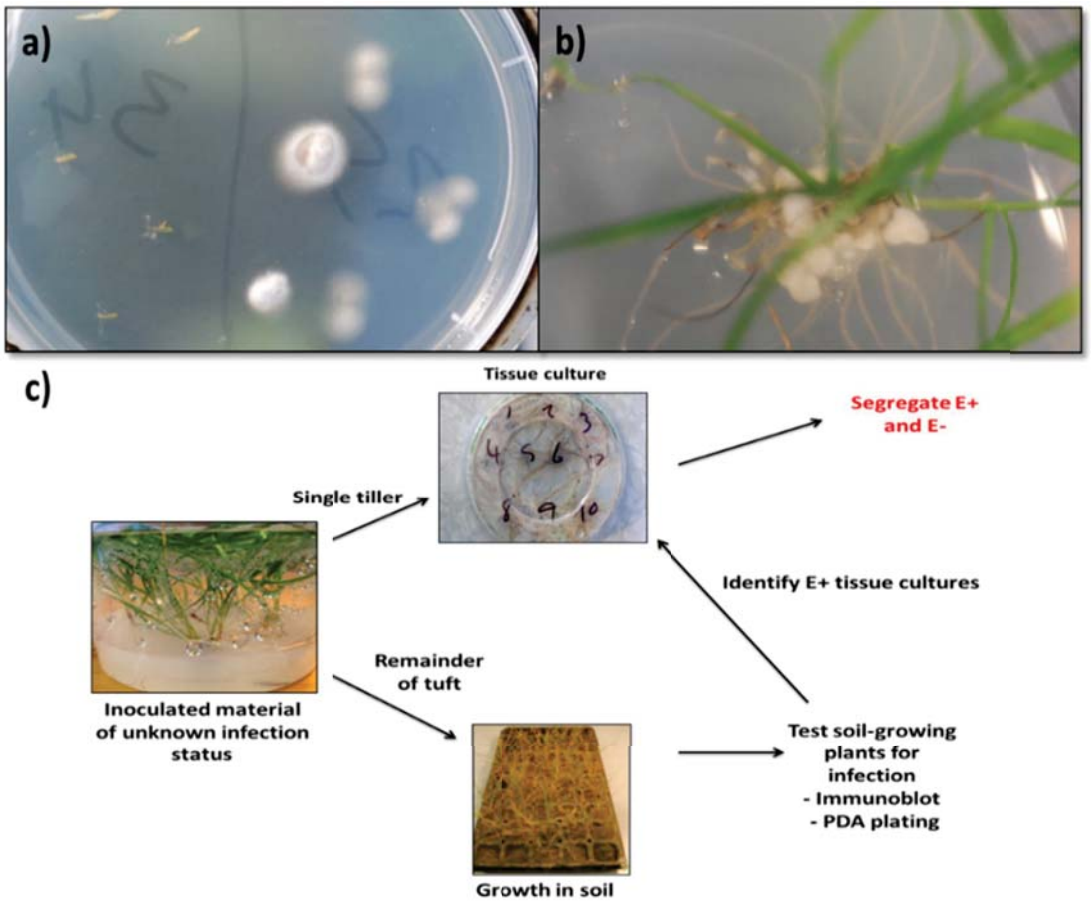


Figure 7. Endophyte isolation and plantlet inoculation. a) PDA plate containing pieces of surface-sterilized pseudostems of inoculated plantlets. **b)** Vertical view of an inoculated plantlet growing on MS media with endophyte culture growing externally on agar at the base of tillers **c)** Schematic representation of strategy used to screen for successfully inoculated plantlets.

2.2.2 Collection and processing of soil

All soil used in both experiments was collected from a ryegrass pasture in Tikokino, (Hawkes Bay, New Zealand) that was included (site number 48) in the '50 pastures project' (Wakelin et al., 2013) (176 27 29.81354 E, 39 49 28.90309 S; World Geodetic System 1984). Soil used in experiment one was collected on the 22nd of March 2016. For experiment two, a small amount of soil was collected from the pasture on the 9th of August 2016 to allow for plantlets to grow to a larger size before introducing them into tubes. Ten days later, enough soil to fill tubes and properly begin the second experiment was then collected from the site. On both occasions, soil was collected to a depth of approximately 20-30 cm, with the top 2-3 cm of turf removed with a knife. Collected soil was stored in 10 L plastic bins

('Envirocrates', Harcor) at 4^{o}C overnight. The following day, soil was passed through a 2 mm sieve to remove large sediment, and was then thoroughly mixed by hand. Given the moisture content of soil collected for experiment two, sieving enough soil to start the experiment took two days. Prior to the beginning of each experiment, 500 g samples (three in experiment one and two in experiment two) were set aside and sent to Hill Laboratories (Hamilton, New Zealand) for physico-chemical analysis- the results of which are shown in Appendix A2.

2.2.3 Determining field capacity of soil

Field capacity (FC) of sieved soil was determined based on the method described in (Klute, 1965). Pre-weighed beakers were filled with 200 g of freshly sieved soil and covered with a piece of cheese cloth secured by a rubber band. Next, soil was completely saturated by being left under running tap water until the beaker was filled with water, before being left sitting upright overnight. The following morning, beakers were put upside down on a wire rack for 48 h to allow the drainage of excess water, and the resulting weight was determined as saturated soil. Saturated soil was then placed in an oven at 105^{o}C for 48 h, and was again weighed with the resulting weight recorded as dry soil. Based on these numbers, FC was calculated using the following equation:

$$Field\ Capacity\ (FC) = \frac{saturated\ soil\ weight - dry\ soil\ weight}{dry\ soil\ weight}\ x\ 100$$

2.2.4 Plant growth

Plant growth tubes and associated equipment used in both experiments is shown in Figure 8. Plants were grown in drainpipes 60 cm in length and 9 cm in diameter that had been vertically cut in half using a vertical band saw (Dyco), sealed back together with 'all clear' silicone (Selleys) and covered with insulation tape (Figure 8 a). The base of each tube was covered with a piece of mulch mat (Coolaroo) secured by a rubber band and insulation tape to allow the drainage of excess water. Tubes were secured to a metal rack (Figure 8 b) with

insulation tape, and were elevated from the ground by a metal rail placed underneath to facilitate drainage of excess water (Figure 8 d). In both experiments, four endophyte-infected and uninfected replicates were included, alongside an identically treated plant-free tube containing only soil. Light measurements were made weekly at canopy height for each tube using a LI-250A light meter with a Quantum Q51097 electrode (Li-Cor), and as plants grew in height the tube holder was re-positioned in the cabinet such that light intensity at canopy height remained at 640 (+/- 80) µmol photons m^{-2} s^{-1} for the duration of each experiment. Plants were grown on a 12/12 h light/dark cycle with temperature during the light cycle 22 +/- 2^{o}C and 12 +/-2^{o}C during the dark cycle, which was monitored by a thermometer placed at canopy height. Humidity within the cabinet was programmed to remain at 70%. After each watering, plants were rearranged on the rack at random to negate any effects due to environmental gradients within the cabinet. During each experiment, three tillers from each replicate were tested by the endophyte-specific immunoblot assay described in section 2.2.1 to confirm their infection status.

2.2.4.1 Experiment one

Tubes were filled with 3.28 kg of freshly sieved soil (4.65 kg at 100% FC). Plantlets tufts growing in agar were removed from media and excised such that each contained three tillers, and roots were also cut back to approximately 2 cm in length before being planted in soil-filled tubes. For the first seven days, plants were watered daily to the rim to gradually saturate soil without drowning plants. Following the seventh day for the remainder of the experiment, plants were placed inside a metal tube holder (Figure 8 c), placed on 10 kg scales (Acurite) and watered to 80% FC three times per week with tap water. However, from day 14 onwards, this estimate was changed based on two additional replicates of FC measurements, and therefore based on the average of all three measurements between day 7 and day 14 plants were watered to approximately 90% FC.

2.2.4.2 Experiment two

Due to plantlets being very small growing in media, fresh soil was collected from the pasture and plantlets were initially grown in root trainers for twelve days in the growth cabinet under experimental conditions, watered every second day to saturation. More fresh soil was then collected from the site prior to moving plants into tubes and properly beginning the experiment. Once tubes were filled with freshly sieved soil it was removed and saturated before being reintroduced to tubes and topped up until approximately 2 cm of space was left at the top of tubes, resulting in a total of 5.45 kg soil at 100% FC added per tube. Prior to potting, plantlets were excised such that each contained three tillers, and roots were cut to approximately 2 cm in length as in experiment one. Plants were watered to estimated saturation by watering tubes which excessively drained multiple times until tubes did not gain any additional weight following watering, and tubes which did not properly drain were watered and left sitting for ten minutes before excess water was decanted from tubes. On day three insects were spotted in the cabinet, and thereafter plants were sprayed weekly with House plant spray (Yates) for three weeks, during which they were watered with *Bacillus thurigensis* (*Bt*) Water (Kiwicare) (two sachets in 20 L of RO water). From day 25 onwards *Bt* treatment and weekly insecticide spraying was discontinued, and plants were thereafter watered with tap water for the remainder of the experiment. On day 28, due to soil progressively slumping further into tubes, tops of tubes were carefully cut using a hand-saw without cutting plants, such that tubes only extended approximately 2 cm above the soil.

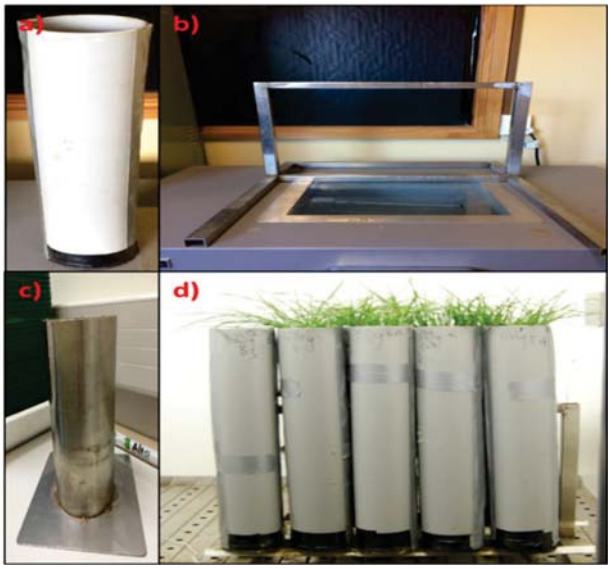


Figure 8. Plant growth tubes and associated equipment. a) Empty plant tube **b)** Metal frame which plant holders were secured to when growing in the cabinet. **c)** Tube holder for stabilizing tubes on scales while being watered **d)** Photo of the interior of the plant growth cabinet with plants secured to the metal frame.

2.2.5 Harvesting and sampling of plants

Plants were harvested after 61 and 68 days in experiment one and two respectively. Plants were not watered for approximately 72 h prior to harvesting to prevent excessive clumping of bulk soil on roots (see appendix A2). Rhizospheres of plants were sampled based on the method described in Edwards et al (2015). Tubes were opened inside of a laminar flow cabinet by removing tape and cutting through silicone with a craft knife. Bulk soil was then removed from root systems by manual shaking and patting using gloves sprayed with 70% ethanol. This process was continued until only soil within approximately 1 mm of the root surface remained intact, and all visible clumps of attached bulk soil had been removed (Figure 9). Gloves were changed between each replicate to prevent cross-contamination. Aboveground plant parts were then separated from roots by excising the base of the apical meristem approximately 5 mm above the root crown, and were set aside in plastic bags and stored at 4^{o}C for subsequent counting of tillers and determination of aboveground biomass. Root systems were placed inside beakers filled with 150 ml of sterile phosphate-buffered saline (PBS) and stirred for 2 minutes using flamed forceps, turning the buffer turbid. For each sample, six 15 ml falcon tubes were filled with turbid PBS and the remainder was discarded. From each plant-containing tube a portion of bulk soil was

collected and stored in a separate falcon tube, and from the bulk soil control tube three falcon tubes were filled with soil collected from subsamples collected throughout the tube. All soil and rhizosphere fractions were stored at -20^{o}C until further processing.

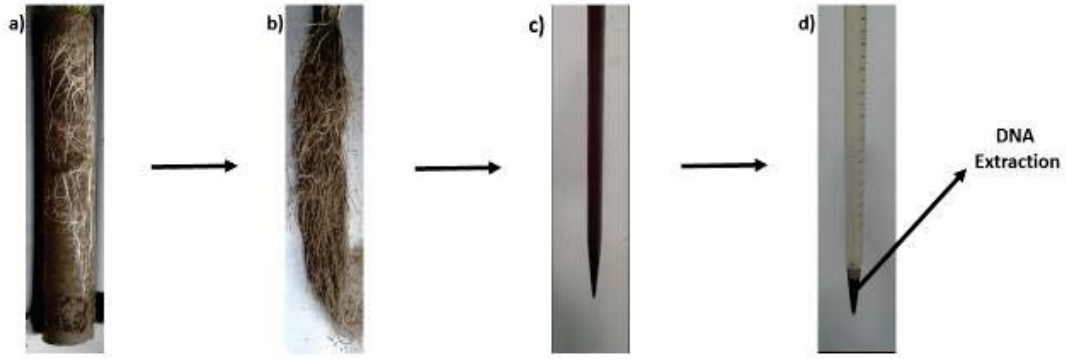


Figure 9. Sampling of plant rhizospheres. a) Plants after removal from tubes with bulk soil still attached. **b)** Root systems after bulk soil had been removed, with rhizosphere soil still attached. **c)** Turbid buffer with rhizosphere soil suspended. **d)** Buffer after centrifugation with rhizosphere soil pelleted.

2.2.5.1 Determination of aboveground biomass

Segmented aboveground plant parts were placed in a beaker and rinsed with tap water to remove residual soil. Aboveground dry biomass of each replicate was determined by oven-drying at 65^{o}C for 48 h, before being weighed using lab scales (Denver).

2.2.6 DNA extraction

Samples were taken from the freezer and allowed to defrost at room temperature for approximately one hour, and rhizosphere fractions were then centrifuged at 2,383 x g for 20 minutes. Supernatant was decanted, and the resulting pellet was defined as rhizosphere soil. Contents of Powersoil (MOBIO) tubes were decanted into sterile 1.5 ml Eppendorf

tubes, and samples of either rhizosphere soil or bulk soil were scooped into empty Powersoil tubes using a small metal spatula. Between samples the spatula was wiped of soil, dipped in 96% ethanol and passed through a Bunsen flame to prevent cross-contamination between samples. Prior to extraction, rhizosphere samples were centrifuged at 10,000 x g for 30 seconds to allow removal of excess buffer with an autopipette, which was repeated for all rhizosphere samples until 0.25 g of soil with excess water removed was contained within each tube. Microbeads and buffer was then decanted back into Powersoil tubes, and DNA extraction was carried out using the Powersoil DNA isolation kit (MOBIO) following the manufacturer's instructions, with the following modification: Mechanical cell lysis (Step 5) was carried out using a MagNAlyser (Roche) processing samples at 5,000 rpm for three minutes, as recommended by Trish Mclenachan (Massey University, Palmerston North). DNA was quantified fluorometrically using the Qubit High Sensitivity assay (Invitrogen), and a 10 µL aliquot of each sample was diluted to 10 ng/µL in 10 mM tris-HCl.

2.2.7 PCR and sequencing

PCR was carried out by amplifying the V4 region of the 16S rRNA gene using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5'-GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) or the ITS1 region of the eukaryotic rRNA gene cluster using the primers ITS1_KYO1 (5'-TAGAGGAAGTAAAAGTCGTAA) and ITS2_KYO2 (5'-TTYRCTRCGTTCTTCATC) (Toju et al., 2012) to assess for prokaryotic and fungal communities respectively. Full primer constructs used also included attached Illumina adapters at the 5' ends of each primer for library preparation (forward primer adapter 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse primer adapter, 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). Reactions were prepared to a total of 25 µL with nuclease-free water (Ambion) as follows: For 16S, reactions contained 200 mM each dNTP, 0.2 µM each primer, 1.75 mM $MgCl_2$, 0.8X Platinum Taq HiFi buffer, 4 µL of Q-solution (Qiagen), 1 U Platinum Taq HiFi polymerase (Invitrogen), and 10 ng of template. ITS reactions were the same as for 16S, however instead contained 0.5 µM of each primer and 2 mM of $MgCl_2$. Thermal cycling was carried out using a Mastercycler (Roche), using

the following conditions: For 16S, an initial 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 45 s, 52^{o}C for 1 min and 72^{o}C for 1 min followed by a final extension at 72^{o}C for 10 min. For ITS, an initial 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 20 seconds 47^{o}C for 30 seconds and 72^{o}C for 20 seconds, followed by a final extension of 72^{o}C for 10 minutes. All PCR reactions for each primer set per sample were carried out in triplicate to minimize stochastic PCR effects, and all three reactions were thereafter pooled and purified using the High Pure PCR product purification kit (Roche) following the manufacturers' instructions, eluted in 50 µL of 10 mM Tris-HCl. Samples were quantified fluorometrically using the Qubit High-Sensitivity assay (Invitrogen), and then ~70 ng of each sample was run on a 1.5% agarose gel to confirm the presence of the correct sized fragments and the absence of significant amplification of non-specific products. For each sample, 16S and ITS amplicons were pooled in equal amounts (within 1 ng of one another) to a total volume of 15 µL. An aliquot of this was then diluted to ~5 ng/µL to a total of 15 µL in 10 mM tris-HCl, and these diluted samples were then submitted to the Massey Genome Service (NZGL) for sequencing. Library preparation was carried out using the Nextera library preparation kit, and all 27 submitted samples (Appendix A5) comprised 35% of a single run on the Illumina MiSeq Platform (Paired-end, 2x250 bp).

2.2.8 Bioinformatics

PhiX control sequences were firstly filtered from raw reads using Bowtie2 (Langmead & Salzberg, 2012) and adapters were removed using the fastq-mcf program of ea-utils suite of tools (https://github.com/ExpressionAnalysis/ea-utils), which was carried out by Mauro Truglio (NZGL). The resulting reads were then processed using the UPARSE pipeline (Edgar, 2013). Paired-end reads were firstly merged using fastq_mergepairs (maximum allowed mismatches in the overlapping region (fastq_maxdiffs)= 12, maximum allowed percentage of mismatches (fastq_maxdiffpct)= 12). 16S (V4) rRNA and ITS1 amplicons were then separated based on their forward primer sequences using fastx_barcode_splitter.pl (FASTX toolkit, hannonlab.com), and were thereafter processed separately (albeit in the same manner unless otherwise specified). Reads were quality-filtered using fastq_filter (UPARSE), discarding reads containing one or more expected errors (maximum allowed

number of expected errors (maxee)= 1.0), and reads passing this filter were then dereplicated using fastx_uniques (UPARSE) using the -sizeout option so that abundances of dereplicated sequences were retained and taken into account during downstream OTU clustering. Operational Taxonomic Unit (OTU) clustering using a 97% similarity threshold, chimera checking and the removal of singletons (-minsize 2) was then carried out using cluster_otus (UPARSE). An OTU table of clustered OTUs was then generated using fastx_global (UPARSE). OTUs were assigned taxonomy using *assign_taxonomy.py* in QIIME (Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010) using the Greengenes (version 13_5) (DeSantis et al., 2006) (assignment method= UCLUST (Edgar, 2010)) and UNITE (version 7) (Koljalg et al., 2013) (assignment method= BLAST (Altschul et al., 1997)) databases for taxonomic assignment of prokaryotic and fungal OTUs respectively. Taxonomic information was then finally incorporated into OTU tables using the biom add-metadata command in QIIME.

Downstream analyses of taxonomically annotated OTU tables were carried out using QIIME. Prior to all analyses (aside from DESeq2 which requires unrarefied OTU tables as input), OTU tables were rarefied using *single_rarefaction.py* such that all samples contained the same number of reads as the sample containing the lowest number of reads (22,838 for prokaryotes and 17,802 for fungi). Rarefaction curves were generated by firstly rarefying OTU tables such that there were ten evenly spaced steps between 10 reads per sample increasing at each rarefaction by 2,280 reads per sample for 16S and 1,700 reads per sample for ITS using *multiple_rarefactions.py* (maximum depth of 22,010 and 17,010 for 16S and ITS samples respectively). Alpha-diversity metrics (number of observed OTUs (metric= 'observed_otus'), Simpson's (Simpson, 1949) Evenness (metric= 'simpson_e')) were then calculated at each rarefaction depth using *alpha_diversity.py*, collated into a single file using *collate_alpha.py*, and finally visualized as rarefaction plots using *make_rarefaction_plots.py*. Chao1 (Chao, 1984) richness estimates of unrarefied samples were also calculated using *alpha_diversity.py*. Beta-diversity analyses were carried out by generating a Bray-Curtis (Bray & Curtis, 1957) dissimilarity matrix using *beta_diversity.py*, creating principal coordinates data of this matrix using *principal coordinates.py*, and finally visualising the coordinates as PCoA plots using EMPeror (Vázquez-Baeza et al., 2013) (*make_emperor.py*). Taxa summary plots were generated using

summarize_taxa_through_plots.py. Filtering of Glomeromycota reads from the ITS OTU table for separate analyses was carried out using *filter_taxa_from_otu_table.py*.

2.2.9 Statistical analyses

Significance of differences in alpha-diversity between different treatments was assessed using *compare_alpha_diversity.py* in QIIME, generating false discovery rate (FDR) (Benjamini & Hochberg, 1995)-corrected *P*-values comparing sample groupings based on Monte Carlo (Wasserstein, 1997) permutations. Statistical significance of differences in beta-diversity were assessed by permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) using *compare_categories.py* in QIIME. For each comparison, dissimilarity matrices were filtered to contain only the two sample groups to be compared using *filter_samples_from_matrix.py*. Significance of differentially abundant OTUs between different sample types was assessed with DESeq2 (Love et al., 2014) via *differential_abundance.py* (QIIME), using unrarefied OTU tables that were filtered of OTUs present in each sample in relative abundances less than 0.1% using a custom python script written by Adam Robbins-Pianka (University of Colorado) (filter_observations_by_sample.py; https://gist.github.com/adamrp/7591573). Prior to each DESeq2 comparison, OTU tables were converted to json format using the biom-convert function, before being filtered of all other samples aside from those in each treatment being assessed using *filter_samples_from_otu_table.py* (QIIME). OTUs present in less than half of all samples in each comparison were also filtered prior to each DESeq2 comparison using *filter_otus_from_otu_table.py* (QIIME).

3 | Results

3.1 Development of experimental design

As outlined in more detail in the introduction (section 1.2), the biological outcomes of a particular host-endophyte interaction are highly variable depending on the specific host cultivar-endophyte strain interaction (Johnson et al., 2007), as well as the conditions under which symbionts are growing (Hesse et al., 2003). Likewise, the plant microbiome has been shown to be dynamic in its taxonomic composition and is collectively shaped by a range of factors including soil type, plant species and even plant genotype (Vandenkoornhuyse et al., 2015). As a result, if effects that *Epichloë* infection have on the rhizosphere microbiome were comparatively small, such impacts could potentially be masked by effects caused by differences in other extraneous variables. To mitigate this, for this study an experimental design was optimized in which plants were grown in conditions that were as identical as possible while also being as representative as possible of conditions plants typically grow under in the field, allowing for consistent differences between infected and uninfected replicates to be more confidently attributed to the presence or absence of the endophyte. The following section describes how this was achieved.

3.1.1 Using plants originating from tissue culture

While the majority of the members of the rhizosphere microbiome originate from the wider bulk soil (Philippot et al., 2013), some are vertically transmitted through seed (Aleklett & Hart, 2013). Furthermore, even small differences in plant genotype have been shown to affect host microbiome structure (Lundberg et al., 2012), as well as the extent to which certain beneficial rhizosphere microbes benefit their hosts (Wintermans et al., 2016). To counteract these effects, all plant material used in this study originated from clonal tissue culture. Due to the recent finding of endophyte-associated impacts towards the root transcriptome in *L. perenne* (NuiD) plants infected with *E. festucae* var. *lolii* (Lp19) (Schmid et al.,2017), this symbiosis was chosen for assessment in this study. Endophyte-infected material was generated by aseptically inoculating the apical meristems of tillers with isolated *E. festucae* var. *lolii* (Lp19) using the method described in Latch & Christensen (1985), and infected material was thereafter segregated from endophyte-negative material

and continually propagated in tissue culture by aseptically separating clusters of tillers into fresh media. This provided genotypically identical sterile endophyte-infected and uninfected plant material for use in plant growth experiments, therefore controlling for these effects.

3.1.2 Introducing sterile plantlets into natural soil

Given that the rhizosphere community originates from the bulk soil, soil type plays a pivotal role in determining rhizosphere community composition (Philippot et al., 2013). To enable our results to be as representative as possible to conditions plants experience in the field, all soil used in this study was collected from a natural ryegrass pasture in Tikokino (Hawkes Bay, New Zealand), that was part of the '50 pastures project' (site 48) (Wakelin et al., 2013). Soil was collected from the site at two different times of the year for each experiment, in March (experiment one) and August (experiment two). Upon introduction of sterile plantlets into soil, it was important that each plant was exposed to the same soil biome to minimize stochastic variation that would occur irrespective of endophyte infection. In nature, microbial communities have been shown to be spatially heterogenous even among seemingly consistent environments (Martirosyan et al., 2013). To mitigate this heterogeneity, soil was firstly sieved to promote a homogenous physical composition, and was then thoroughly mixed by hand.

3.1.3 Growing plants in controlled conditions in a growth cabinet

To control for effects due to variability in environmental conditions, in this study plants were grown in a growth chamber under controlled conditions (see section 2.2.4). Given the elevated 60 cm height of tubes that plants were grown in, programming the cabinet to the desired settings did not necessarily result in this being present at canopy height. Therefore, light and temperature measurements were manually monitored using a thermometer kept at canopy height and by measuring light intensity weekly at canopy height using a light meter.

3.1.4 Sampling the rhizosphere

A range of different strategies have been used in previous studies to sample the rhizosphere. Some studies define rhizosphere soil as soil which falls from the root surface when loosely shaken (Wakelin et al., 2015), whereas other have instead removed all loosely associated soil from the roots and sampled the rhizosphere by washing roots in buffer, centrifuging the buffer and finally extracting DNA from the resulting pellet (Bulgarelli et al., 2015; Edwards et al., 2015). To enrich for rhizosphere soil as much as possible, in this study the latter approach was used (see Figure 9).

3.1.5 Selection of PCR primers

A wide variety of primers are routinely used in metabarcoding studies. No known primer pairs are completely universal, and some of the widely used prokaryotic and fungal primers have been shown to be biased towards annealing to the templates of certain phyla, which can falsely inflate abundance estimates (Bellemain et al., 2010; Klindworth et al., 2012; Lindahl et al., 2013). Primer choice is therefore an important consideration and should be made depending on the particular aims of a given study. The following section gives a general description of known characteristics of the primer pairs chosen for use in this study, before explaining the reasoning behind their use.

3.1.5.1 Prokaryotic 16*S* rRNA primers 515F/806R

The prokaryotic 16S ribosomal RNA (rRNA) gene contains nine hypervariable regions (Gray et al., 1984), providing a variety of potential primer targets for metabarcoding studies assessing prokaryotic microbial diversity. Studies comparing the results of these primers targeting different hypervariable regions have consistently shown that the V4 region is a robust choice (Ghyselinck et al., 2013; Peiffer et al., 2013), although a variety of different regions are commonly targeted, no currently known primer pair provide universal coverage. As a result, the use of a wide variety of target regions complicates the comparison of results of different studies that have used different primers. To mitigate this

problem, there have been initiatives developed that urge researchers to adopt protocols proven to be effective as a means of standardization, such as the Earth Microbiome Project (EMP)(Gilbert et al., 2014). Two recent high-throughput analyses assessing the rhizosphere microbiome of tall fescue in response to *Epichloë coenophiala* infection used the primers described in the EMP standard 16S protocol (515F/806R), which amplify the V4 region (Roberts & Ferraro, 2015; Rojas et al., 2016). To enable our results to be comparable to the results of these similar studies, this primer pair was chosen for use in this study.

3.1.5.2 Fungal ITS1 primers ITS1-F_KYO2/ITS2_KYO2

Community analyses of fungi that have been so far undertaken have targeted a range of regions of the rRNA gene cluster including the small subunit, the large subunit (Gottel et al., 2011; Shakya et al., 2013), and the internal transcribed spacer (ITS) region (McGuire et al., 2013). The ITS region has the advantages of being highly variable and exhibiting low intraspecific variation, however this high level of variation means that the ITS region is a poor indicator of phylogenetic distances between different taxa at higher taxonomic levels. In contrast, the small-subunit/large subunit (SSU/LSU) regions contain conserved regions which allow alignment across long phylogenetic distances, however provides diminished phylogenetic resolution at lower taxonomic ranks compared with the ITS region (Lindahl et al., 2013). Despite the targeting of each region having their advantages and disadvantages, given that the ITS region was shown as having the highest probability of correctly identifying the broadest range of fungi it has been proposed as the official 'universal barcode' for fungi (Schoch et al., 2012). Primers which targeted the ITS region that were used in early fungal community analyses have since been illustrated as being biased toward specific sub-groups of fungi (Bellemain et al., 2010). To ameliorate this, Toju et al (2012) designed primers which have broad coverage across Basidiomycetes and Ascomycetes, while excluding plant sequences and being seemingly unbiased towards any particular fungal groups. The primers were also shown to have broad coverage across Glomeromycota and other non-Dikarya (Toju et al., 2012). Given that a recent comparison of primers flanking the ITS1 and ITS2 regions found the ITS1 region to have higher species discrimination to the genus level than the ITS2 region, (Wang et al., 2015), in this study the ITS1 region was amplified using the primers ITS1-F_KYO2 and ITS2_KYO2 (Toju et al., 2012).

3.2 No significant effect of endophyte infection on plant growth

Once both successfully inoculated and uninoculated plantlets had propagated in agar such that there were at least four E+ and E- tufts each containing at least three tillers, fresh soil could be collected from the site and the first plant growth experiment could commence. Two plant growth experiments were carried out in this study, each comparing four endophyte-infected plants with four uninfected plants growing in soil collected from the pasture at two separate times of the year.

Prior to the beginning of each experiment, samples of freshly sieved soil were sent for physico-chemical analysis, the results of which are shown in Appendix A2. Between experiments, physico-chemical profiles of the soil were almost identical aside from soil collected in experiment two having a slightly higher pH (5.2 in E1 versus 5.5 in E2), and slightly lower phosphorus levels (109 mg/L in E1 versus 97 mg/L in E2) than in the first experiment. Notably, phosphorus levels in the soil were approximately 3-3.5 times as high as what is typically considered as being 'high' levels of phosphorus in agricultural soils (Soil Tests & Interpretation, Hill Laboratories). Moisture content of collected soil following sieving (as determined based on field capacity measurements) was also higher in the second experiment than the first (18% in E1 versus 27% in E2).

3.2.1 Experiment one

Soil was collected from the ryegrass pasture on the 22nd of March 2016, and stored at 4^{o}C overnight. The following day, the soil was passed through a 2 mm sieve to remove large sediment and was then mixed thoroughly by hand. Tubes were then filled with 3.38 kg of the freshly sieved soil, as this was the amount required to completely fill tubes leaving ~2 cm of space at the top. Plantlet tufts consisting of three tillers (four replicates for each treatment) were then removed from agar and roots were cut to ~2 cm using a sterile scalpel. Plantlets were then planted into soil-filled holders and were placed into the growth cabinet secured to a metal rack (see Figure 8 d).

Given that the experiment began with freshly sieved soil that was relatively dry (~30% field capacity (FC)), for the first ten days tubes were watered daily to the rim to allow soil to gradually saturate without flooding plants. Following this, for the remainder of the experiment plants were watered to 80% FC three times per week. However, as the soil became saturated it gradually slumped further (~3-5 cm) down into tubes. While this complicated the accurate counting of tillers in this experiment, it did not seem to have any negative effects on the growth of any of the plants.

Throughout the experiment small plants would occasionally sprout up from the soil- presumably from seeds present, which had not been treated aside from sieving. To prevent these from having any impacts towards plants of the experiment, tubes were inspected three times per week and invading plants were removed whenever identified.

3.2.2 Experiment two

Due to the small size of plantlets growing in media, in experiment one some of the tillers introduced into each tube died during the first week of the experiment (Figure 10 a). To prevent this from also occurring in the second experiment, on the 9th of August 2016 a small amount of fresh soil was collected from the pasture, sieved, and then plantlets were grown in root trainers for twelve days in the growth cabinet, watered every second day to saturation. Fresh soil was again collected from the site of the 19th of August 2016 prior to tubes being filled and beginning the experiment. Upon collection, soil was wetter than in the first experiment, causing the sieve to continually become clogged while sieving. As a result, sieving enough soil to begin the experiment took two days, and during this time the soil was kept at 4^{o}C overnight. Unlike experiment one however, soil was saturated prior to being introduced into the tubes with the expectation that this would reduce the slumping of soil within tubes that was observed in experiment one, and would also allow use of the desired watering regime from the very beginning of the experiment. This resulted in each tube containing a total of 5.45 kg of soil at 100% FC, meaning that the soil was more compact than in the first experiment where only 4.65 kg of soil at 100% FC was added per tube. On the 21st of August 2016 plants were removed from root trainers, roots were cut back to ~2 cm in length and tufts were excised such that each contained three tillers. Tufts

(4 infected and 4 uninfected) were then placed in 8 soil-filled tubes and grown under the same conditions as in the first experiment.

It was not possible to adhere to the intended watering regime in this experiment, as some of the tubes drained excessively as soon as they were watered while others did not drain at all and instead quickly became waterlogged. As a result, some tubes did not retain enough water to reach the estimated 80% field capacity even after being watered multiple times, while others would overflow before reaching this weight. To mitigate this, tubes which drained excessively were watered and allowed to drain multiple times until their weight remained constant following drainage, while tubes which did not drain were saturated with water and after ~10 minutes excess water was poured off. Following this each of these tubes weighed between 5.2 and 5.5 kg, and therefore tubes which drained normally were watered until they were also within this weight range. Due to the soil of excessively draining tubes drying out observably quicker than in the first experiment, in this experiment plants were instead watered every second day.

Despite soil being saturated prior to being packed into tubes, the gradual slumping of soil within tubes that was observed in experiment one also eventually occurred in experiment two. To mitigate this and improve the accuracy with which tiller numbers could be counted, on day 24 the tops of tubes were carefully cut with a handsaw without cutting plants such that the tops of tubes only extended ~1-2 cm above the soil.

An additional difference between this experiment and experiment one resulted from insect infestation. On day three, small black insects that were probably fungus gnats were spotted flying within the cabinet. To adhere to regulations surrounding the use of the facility in which the plant growth chamber was housed, immediate insecticide treatment was mandatory. For the following three weeks, plants were sprayed weekly with Yates 'natures way' house plant spray (active ingredients- 0.19 g/L pyrethrum, 0.7 g/L piperonyl butoxide, 18 g/L hexane), and were watered with RO water to which *Bacillus thuringiensis* subspecies Kurstaki (9.25 million spores/L) had been added. However, addition of these spores in the second experiment did not increase the relative abundances of *Bacillus* present at the genera level between sample of each experiment (2 ± 0.2% in experiment one versus 1.7 ± 0.3% in experiment two), or any OTUs assigned as *Bacillus* (Appendix A3). Sticky traps were scattered around the cabinet, and insecticide treatment was discontinued when no new

insects were found in the traps for three consecutive days. While numbers of insects were not recorded, these were not very high (usually <5 new insects spotted in traps per day) and were mostly spotted on the walls of the cabinet as opposed to being found on plants. Plants also displayed no symptoms of insect herbivory.

3.2.3 State of plants at the time of sampling

During preliminary trials, it was found that sampling plant roots after plants had recently been watered resulted in excessive clumping of soil over roots, which made the sampling of rhizosphere soil difficult. To prevent this, plants were not watered for ~72 h prior to harvesting.

Plants were harvested after 61 and 68 days of growth for experiment one and two respectively. Weekly monitoring of tiller numbers throughout each experiment showed that in both instances plants were exponentially tillering when harvested (Figure 10). Aboveground biomass measurements for each replicate at the time of harvesting are shown in Table 1. In both experiments, comparison of the four E+ and E- plants using the Mann-Whitney U test showed no significant endophyte effect on total number of tillers per tube (E1, 46 ± 8 in infected versus 39 ± 8 in uninfected plants; E2, 49 ± 16 versus 38 ± 10), aboveground biomass (E1, 3.12 ± 0.8 g versus 3.35 ± 0.4 g; E2, 2.36 ± 1.06 g versus 1.92 ± 0.5 g) or average tiller weights (E1, 67 ± 7 mg versus 92 ± 31 mg; E2, 47 ± 12 mg versus 51 ± 4 mg). Comparison of all 8 plants of the first experiment with those of the second found no significant differences in number of tillers (42 ± 8 versus 43 ± 13), however plants of experiment one had significantly higher aboveground dry biomass (3.2 ± 0.6 g versus 2.1 ± 0.8 g; P= 0.007) and average tiller weights (79 ± 25 mg versus 49 ± 8 mg; P= 0.007) than in the second experiment.

Root biomass of replicates was also to be recorded, however the very fine morphology of the plant roots coupled with the high clay content of the soil meant that it was virtually impossible to remove soil from roots while keeping root systems completely intact. Due to this, in this study root biomass was not recorded. However, while it was readily apparent that root systems of the first experiment were considerably larger than in the second, there

were no morphological differences observed in root systems of infected and uninfected plants in either experiment (Figure 11).

Table 1. Aboveground biomass of replicates at the time of harvesting.

Experiment[1]	Infection[1] status	Replicate[2]	Number of tillers	Aboveground dry biomass (g)[3]	Average tiller weight (mg)[4]
1 42.1 ± 8.4 3.2 ± 0.6 79.1 ± 24.7	**E+** 45.7 ± 7.9 3.1 ± 0.9 66.8 ± 7	1	57	4.33	75
		2	45	3.06	68
		3	39	2.29	58
		4	42	2.80	66
	E- 38.5 ± 8.3 3.4 ± 0.4 91.5 ± 31.2	1	45	3.47	77
		2	44	2.79	63
		3	38	3.49	91
		4	27	3.65	135
2 43 ± 13.4 2.1 ± 0.8 48.9 ± 8.3	**E+** 48.5 ± 15.5 2.4 ± 1.1 47 ± 11.6	1	33	1.24	37
		2	70	3.66	52
		3	47	1.81	38
		4	44	2.72	61
	E- 37.5 ± 9.7 1.9 ± 0.5 50.8 ± 4.3	1	44	2.04	46
		2	26	1.28	49
		3	47	2.48	52
		4	33	1.86	56

[1] Averages and standard-deviations are shown of each colour-coded growth parameter within each experiment/endophyte status category.

[2] Replicates highlighted in red represent those that were not analysed further.

[3] Determined by oven drying at 65°C for 48h.

[4] Average tiller weight was estimated by dividing the total number of tillers by the aboveground dry biomass of each replicate.

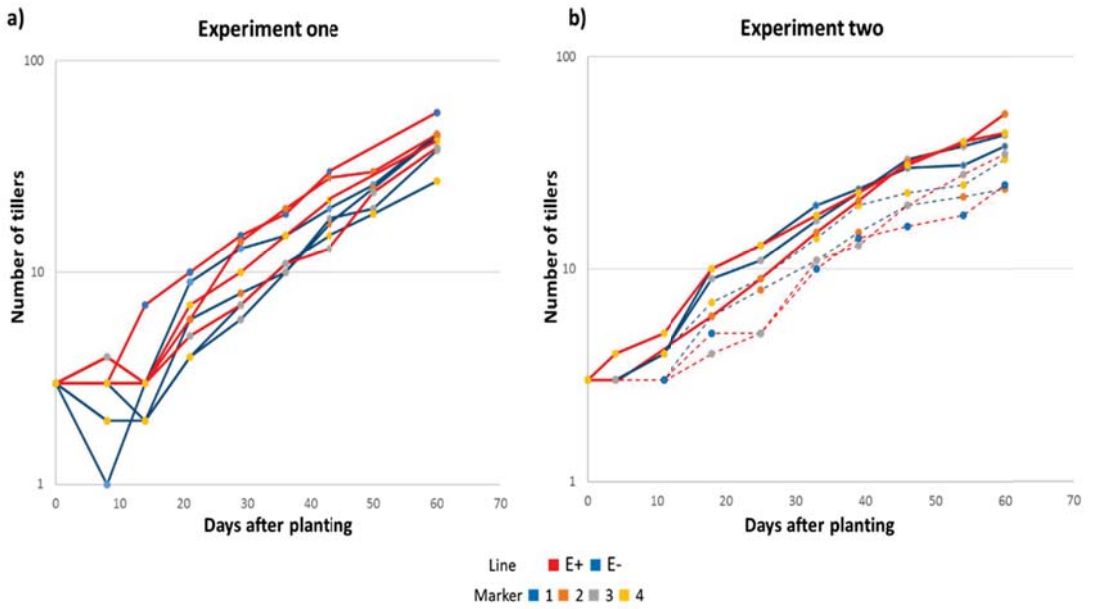


Figure 10. Plant tillering rates. a) experiment one, and **b)** experiment two. Blue lines= E-, Red lines= E+. Markers are coloured differently depending on their corresponding replicate number. Dashed lines in experiment two represent plants whose root microbiomes were not analysed.

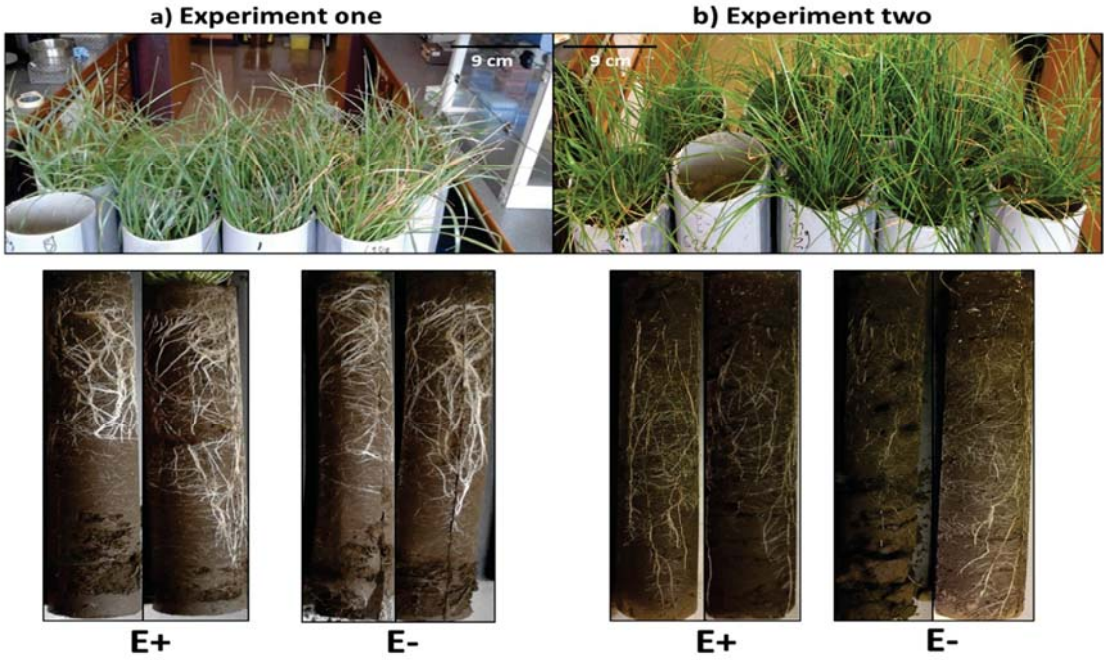


Figure 11. Plants at the time of harvesting. Photo of plants and bulk soil control tube immediately before harvesting of **a)** experiment one and **b)** experiment two, with photos of root systems of two infected (E+) and uninfected (E-) replicates from each experiment underneath.

3.3 DNA extraction

DNA yields were typically twice as high in rhizosphere samples compared with bulk soil samples (E1, Average of 26.6 ± 3.2 in bulk soil versus 47 ± 5 ng/µL in rhizosphere samples; E2, Average of 43.2 ± 5.8 in bulk soil versus 72 ± 2.9 ng/µL rhizosphere samples), aligning with the general consensus of there being higher microbial biomass in the rhizosphere than in bulk soil (Philippot et al., 2013). However, there were no apparent effects of endophyte infection on the DNA yields of rhizosphere samples in either experiment (Appendix A4).

3.4 PCR

Examples of Purified 16S (V4) and ITS1 amplicons are shown in Figure 12. Note that in 16S samples a faint ~750 base pair (bp) band was visible, however this small proportion of sequenced fragments corresponding to these non-specific amplicons would have been filtered out during the merging of paired-end reads, as reads from each end would not have overlapped. For ITS1 amplicons, the smearing above the main ~320 bp fragment was expected, as the length of the ITS1 region varies between different fungal species (Bellemain et al., 2010; Toju et al., 2012).

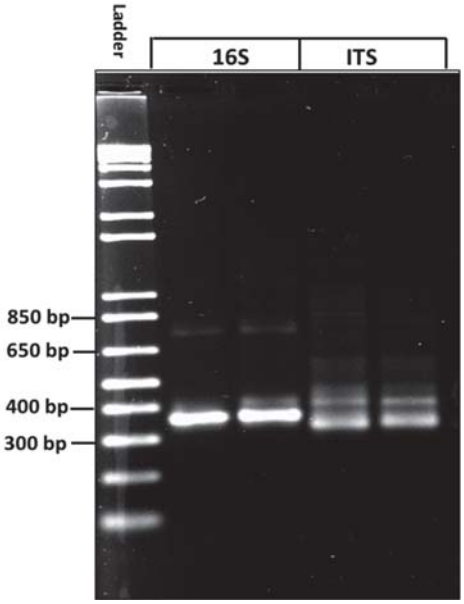


Figure 12. Purified 16S and ITS amplicons. Example of two 16S and ITS samples (~70 ng each).

3.5 Bioinformatic analyses

Once PCR amplicons had been generated and purified, they were submitted to the Massey Genome service for high-throughput sequencing on the Illumina MiSeq platform. The 27 samples (Appendix A5) resulted in a total of 1,228,409 16S reads and 1,269,718 ITS1 reads. Forward and reverse reads had an average overlap of 212 bp. As recommended by the USEARCH documentation (see https://www.drive5.com/usearch/manual/) for instances where there is a large overlap in forward and reverse reads, the number of allowed mismatches in the overlapping region was therefore increased from 5 to 12 (fastq_maxdiffs=12), as well as the percentage of differences allowed increased to 12% (fastq_maxdiffpct=12). This resulted in the percentage of total reads successfully merged to increase from 50.6% to 70%. Quality-filtering and Operational Taxonomic Unit (OTU) clustering using a 97% similarity threshold assigned these to a total of 3,868 prokaryotic and 3,103 fungal OTUs.

Numbers of reads and OTUs per sample in raw OTU tables are shown in Figure 13. To estimate the coverage rates of OTUs detected at these sequencing depths, for each sample of the unrarefied OTU table the Chao1 richness estimator (Chao, 1984) was used to estimate the total number of OTUs predicted to be present in each sampled environment. Based on the actual number of OTUs detected in each sample, coverage rates were calculated by dividing the number of observed OTUs by the Chao1 estimates (Appendix A5). Based on this, coverages of OTUs of unrarefied samples were 82.9 ± 3.9% for prokaryotes and 81.7 ± 5.3% for fungi.

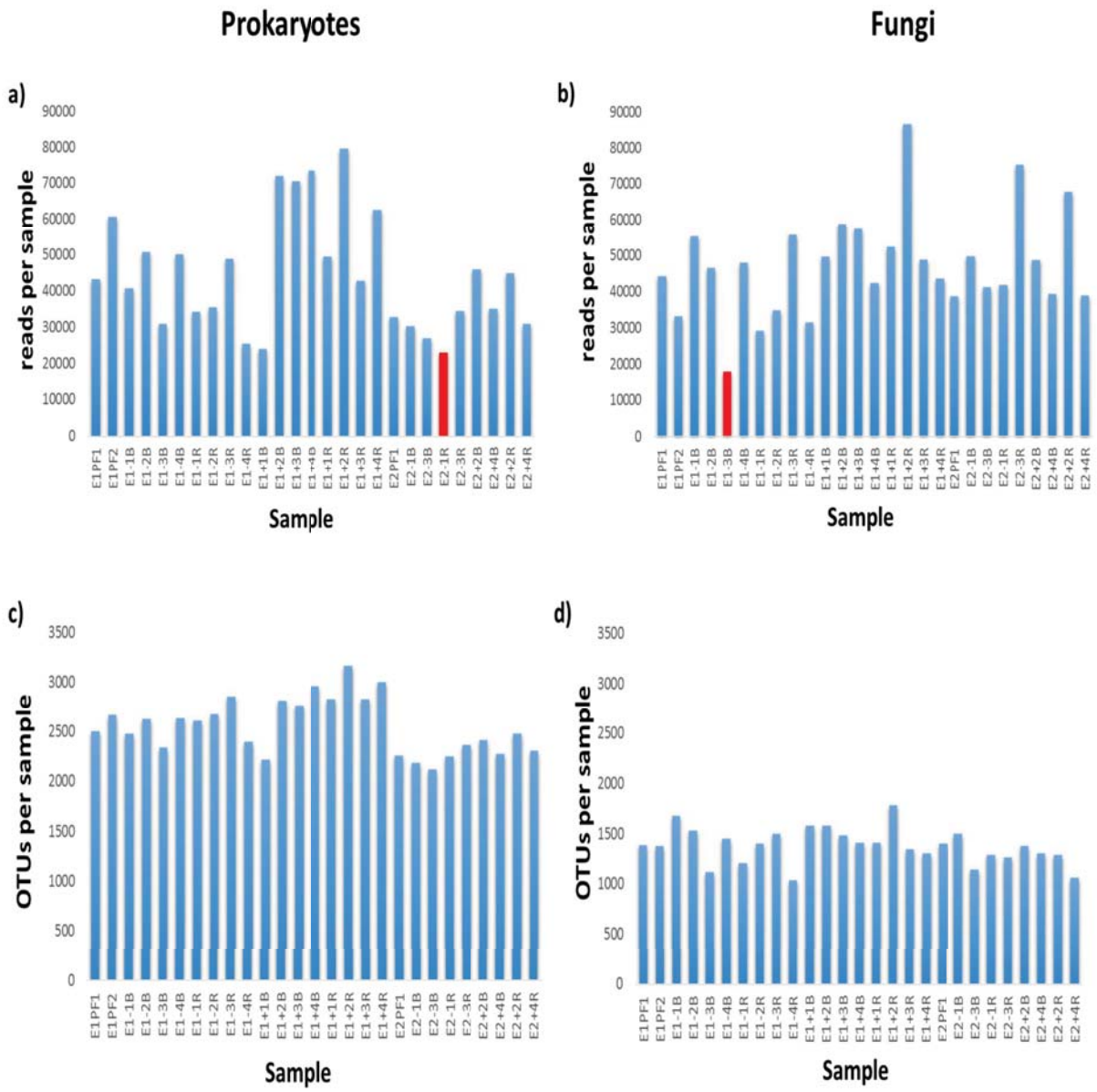


Figure 13. Numbers of reads and OTUs per sample in unrarefied OTU tables. The sample containing the lowest number of reads in each dataset to which all other samples were rarefied to are highlighted in red. Samples are labelled as- E1/E2= experiment one/two, +/-= Endophyte-positive/negative, PF= plant-free, B/R= Bulk soil/Rhizosphere. Numbers in each sample name denote the replicate number.

3.5.1 Normalization of sequencing depths using rarefaction

Raw OTU tables consist of samples containing unequal numbers of reads (Figure 13 a, b). To allow valid comparisons to be made between samples, these differences in sequence depth require normalization. One widely used means of normalization is to rarefy the data,

which involves the random subsampling of each sample such that a new OTU table is generated in which all samples contain the same number of reads. As the lowest number of counts in the samples included in this study were 22,838 for prokaryotes and 17,802 for fungi (Figure 13 a, b; Appendix A5), each OTU table was therefore rarefied to these depths. The resulting normalised OTU tables were thereafter used in downstream analyses (aside from DESeq2 analyses, as this required a non-rarefied table as input).

To assess the adequacy with which the sequencing depth of each rarefied dataset analysed in this study represented the total diversity within each community, rarefaction curves were generated using both the total number of OTUs as a measure of species richness, as well as the Simpson's evenness (E) index a measure of species evenness (Figure 14). Simpson's E is calculated by firstly calculating the Simpson's (D) index (Simpson, 1949), and then expressing this as the proportion of the maximum value of D if all members of the population were evenly distributed. Therefore, a Simpson's E index of 1 represents complete evenness, and the closer the index is to zero the more uneven the OTU abundances are within the sample. Firstly, normalised OTU tables were rarefied to a range of depths between 10 sequences per sample and the maximum rarefaction depth of each rarefied OTU table, and both species richness and evenness metrics were calculated at each depth and then plotted as a curve. For observed OTUs, at the highest rarefaction depth curves had still not completely plateaued (Figure 14 a, c), indicating that there were still novel OTUs being detected at this sequence depth. Rarefying OTU tables to 70,000 reads per sample and generating rarefaction curves (using the few remaining samples containing this number of reads) showed that even at this rarefaction depth curves of the total number of OTUs had still not completely plateaued (Appendix A6), emphasizing the hyper-diversity of prokaryotic and fungal taxa residing in the soil used in this study. On the other hand, for both prokaryotes and fungi the Simpson's E index appeared to have saturated at this depth (Figure 14 b, d), indicating that deeper sequencing would not have been likely to alter estimates of species evenness of samples.

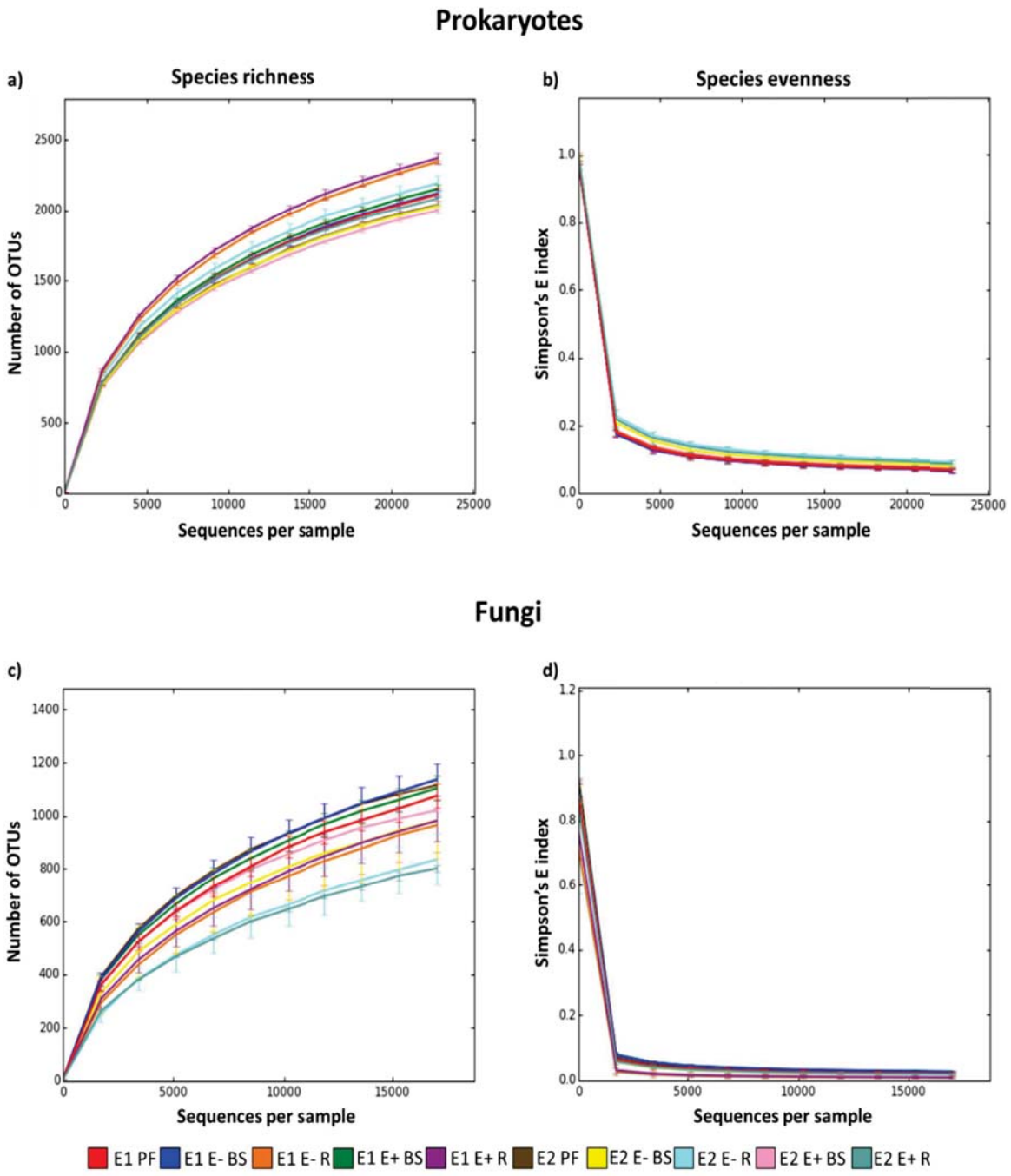


Figure 14. Rarefaction curves of rarefied OTU tables. Rarefaction curves of both total number of OTUs and Simpson's E metric for prokaryotes (a, b) and fungi (c, d). E1/E2= Experiment one/two, PF= Plant-free, BS= Bulk soil, R= Rhizosphere. Error-bars indicate standard deviations.

To estimate OTU coverage rates in the rarefied dataset, the number of OTUs present in rarefied samples was divided by the Chao1 (Chao, 1984) richness estimate obtained for each respective unrarefied sample (Appendix A5). Coverages for samples ranged between

64% to 77% for prokaryotes with an average of 71%, and between 48% and 70% for fungi with an average of 61.3%. However, despite the sequencing depths used in this study not capturing the estimated entirety of OTUs present in samples, the depths used should nevertheless provide an adequate representation of more abundant taxonomic groups present in each sample (Rojas et al., 2016).

3.5.2 Alpha-diversity analyses

To assess for differences in levels of diversity within samples, alpha-diversity (Whittaker, 1972) analyses were conducted in QIIME (Caporaso et al., 2010). Statistical analyses of differences in species richness and evenness between sample groupings were conducted using a test based on the two-sample *t*-test, however *P*-values were instead calculated permutationally using Monte Carlo (Wasserstein, 1997) simulations rather than by referring to the *t*-distribution. Given the finding of Roberts & Ferraro (2015) of increased species richness in the rhizosphere microbiome of *E. coenophiala*-infected tall-fescue plants compared with uninfected plants, it was hypothesized that prokaryotic species richness may also be increased in infected plants in this symbiosis. Given the common observation of species richness decreasing in each compartment of the root microbiome with increasing proximity to the root (Bulgarelli et al., 2013; Vandenkoornhuyse et al., 2015), it was also hypothesized that bulk soil samples would contain higher levels of species richness than rhizosphere samples.

3.5.2.1 Prokaryotic and fungal species richness of the rhizosphere microbiome is not significantly affected by endophyte infection

To assess for the relative differences in species richness of the rhizosphere of endophyte-infected and uninfected plants, box-plots comparing species richness between samples grouped by experiment, sample type and endophyte status were generated (Figure 15 e, f). In both experiments, endophyte infection had no significant effect on species richness of prokaryotic nor fungal communities in the rhizosphere. However, there was an apparent but non-significant decrease in prokaryotic species richness in the rhizosphere of infected

plants in the second experiment (2084 ± 41 OTUs in E+ versus 2191 ± 55 OTUs in E-, *P*= 0.53).

3.5.2.2 Higher prokaryotic species richness in the rhizosphere than in bulk soil

Unexpectedly, in both experiments prokaryotic species richness was significantly higher in the rhizosphere than in the bulk soil (E1, 2136 ± 33 versus 2357 ± 31 OTUs, (*P*= 0.015); E2, 2015 ± 18.7 versus 2138 ± 71.9 OTUs, (*P*= 0.05)) (Figure 15 c). On the other hand, fungi followed the expected pattern of having higher species richness in the bulk soil than in the rhizosphere in both experiments (E1, 1121 ± 55 versus 974 ± 91 OTUs; E2, 1002 ± 104 versus 818 ± 71 OTUs)- although this difference was only significant in the first experiment (E1, *P*= 0.045; E2, *P*= 0.16) (Figure 15 d).

3.5.2.3 Differences in species richness between each experiment irrespective of endophyte infection

Regardless of sample type or infection status, both prokaryotic and fungal species richness were significantly lower in the second experiment than in the first (Prokaryotes, 2231 ± 117 in E1 versus 2072 ± 77 OTUs in E2, (*P*= 0.002); fungi, 1051 ± 101 in E1 versus 933 ± 137 in E2, (*P*= 0.025) (Figure 15 a, b). Overall, the bulk soil of experiment two had an observable but non-significantly lower species richness than in experiment one. Despite the difference in alpha-diversity between each experiment, in both instances the same general trends of higher diversity in the rhizosphere than bulk soil (or vice-versa for fungi) were observed. There were also no discernible differences between bulk soil samples originating from plant-containing tubes compared with those from plant-free controls, suggesting that presence of the plant does not significantly stimulate diversity of the wider bulk soil (Figure 15 c, d).

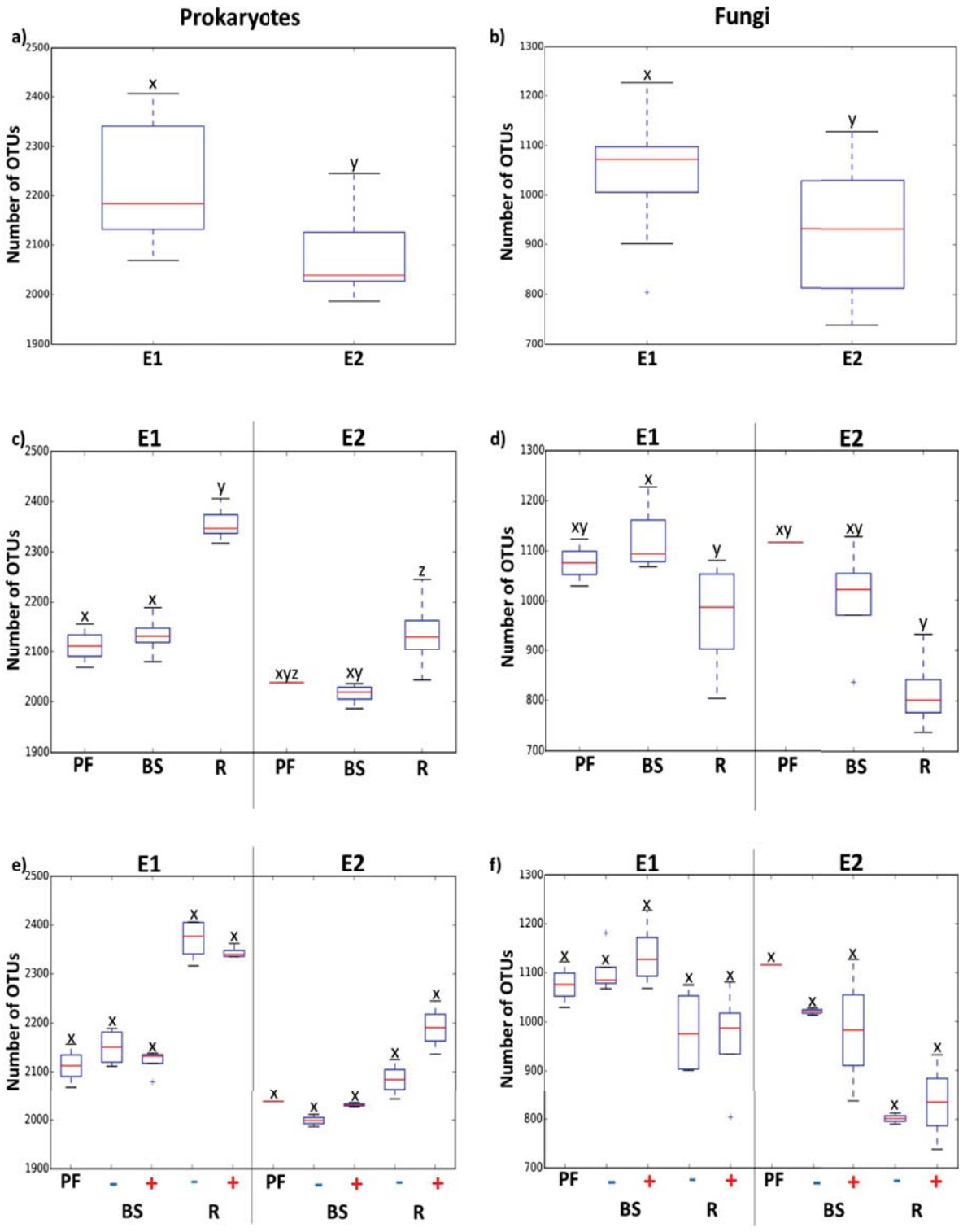


Figure 15. Species richness comparisons. Box-plots of the number of OTUs present in samples grouped by experiment (a, b), experiment and sample type (c, d), and by experiment, sample type and endophyte treatment (e, f). E1/E2= Experiment one/two, PF= Plant-free, BS= Bulk soil, R= Rhizosphere. Categories containing a different letter above them were statistically significant ($P \leq 0.05$; FDR-corrected) from one another.

3.5.2.4 No significant effect of endophyte infection on species evenness of the rhizosphere microbiome

Box-plots of species evenness of samples clustered by experiment (a, b), experiment and sample type (c, d) and experiment, sample type and endophyte status (e, f) are shown in Figure 16. Overall, for both prokaryotes and fungi all samples displayed high levels of unevenness, with little variability in this metric between different samples (between 0.060 and 0.101 for prokaryotes and between 0.004 and 0.033 for fungi). Nevertheless, irrespective of sample type, prokaryotic species evenness was significantly higher in samples of the second experiment than in the first (0.070 ± 0.006 in E1 versus 0.084 ± 0.010 in E2, (*P*= 0.001)) (Figure 16 a). Comparison of samples based on experiment and sample type found that prokaryotic rhizosphere samples of experiment two had significantly higher species evenness than rhizosphere samples of the first experiment (0.072 ± 0.008 in E1 versus 0.093 ± 0.008 in E2, (*P*= 0.038). For fungi, there was a lower species evenness in the rhizosphere than in bulk soil in both experiments, although this difference was only significant in the first experiment (E1, 0.027 ± 0.003 in BS versus 0.009 ± 0.003 in R, (*P*= 0.03); E2, 0.02 ± 0.005 in BS versus 0.01 ± 0.007 in R (*P*= 0.3)) (Figure 16 c). However, while there was a discernible decrease in species evenness in the fungal rhizosphere microbiome of infected compared with uninfected plants in the second experiment (0.020 ± 0.003 in infected versus 0.008 ± 0.003 in uninfected, (*P*= 0.55)), there were no apparent nor significant differences between prokaryotic species evenness in the rhizosphere of infected and uninfected plants in either experiment (Figure 16 e).

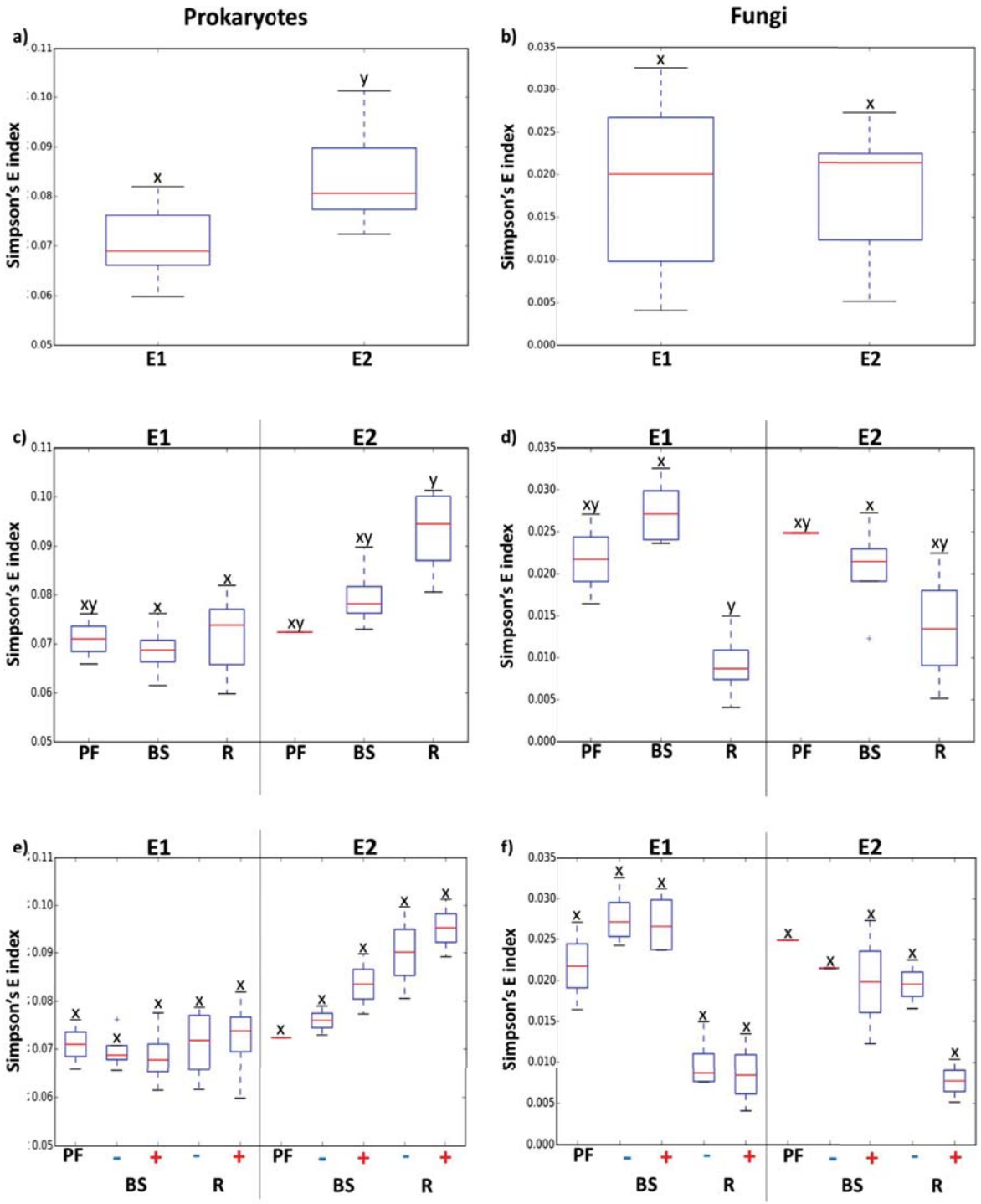


Figure 16. Species evenness comparisons. Samples clustered by experiment (a, b), experiment and sample type (c, d), and experiment, sample type and endophyte status (e, f). E1/E2= Experiment one/two, PF= Plant-free, red crosses/blue dashes= Endophyte positive/negative, BS= Bulk-soil, R= Rhizosphere. Categories containing a different letter above them were statistically significant ($P \leq 0.05$; FDR-corrected) from one another.

In summary, these results show that in both experiments neither prokaryotic nor fungal alpha-diversity of the rhizosphere microbiome were significantly affected by endophyte infection. Irrespective of endophyte infection, samples of experiment one had significantly higher species richness than the second experiment, and within each experiment prokaryotic species richness was significantly higher in the rhizosphere than in bulk soil. The opposite trend was observed in both experiments for fungi where there was instead higher diversity in the bulk soil than in the rhizosphere, although this difference was only significant in the first experiment. There were no significant effects of endophyte infection on species evenness of prokaryotic and fungal rhizosphere microbiomes in either experiment, although in the second experiment there was an apparent increase in species evenness of fungal communities in the rhizosphere of uninfected compared with infected plants in the second experiment but not the first.

3.5.3 Beta-diversity analyses

In addition to assessing for differences in diversity within samples, beta (between-sample)-diversity (Whittaker, 1972) analyses were conducted to visualize the relative differences in community composition between samples. While the main objective was to assess for differential clustering of rhizosphere samples of infected and uninfected plants, this analysis also allowed for the assessment of dissimilarity in the community composition of communities in bulk soil versus rhizosphere samples, as well as between samples of each experiment. This was assessed by generating Bray-Curtis (Bray & Curtis, 1957) dissimilarity matrices, which is a statistical tool used to quantify the compositional dissimilarity between samples based on counts in each sample. The dissimilarity matrix ranges between 0 and 1, where 0 indicates samples share all the same species present in the same abundances, whereas 1 indicates that the samples do not share any species. Principal coordinates were then generated based on this matrix, and displayed as principal coordinate analysis (PCoA) plots. Statistical significance of differential clustering of different sample groupings on the resulting plots was then calculated using PERMANOVA (Anderson, 2001). This involves the permutational calculation of the Pseudo-F statistic, which is defined as the ratio of within-treatment clustering versus between-treatment

clustering of groups of samples. Therefore, the higher the Pseudo-F statistic is the more likely it is that the null hypothesis of there being no difference in locations of the two tested treatments on the plot is false (Anderson, 2001). Despite alpha-diversity not being affected by endophyte infection, it was hypothesized that community composition could still be affected in terms of the composition and/or relative abundances of OTUs in each sample.

3.5.3.1 Mild endophyte-associated shift in the prokaryotic rhizosphere community in the first experiment but not the second

In both experiments, prokaryotic rhizosphere communities appeared to cluster together irrespective of endophyte infection (Figure 17 a). However, PERMANOVA analyses found that the minor partitioning of rhizosphere samples of infected and uninfected plants (Pseudo-F= 1.25) was statistically significant (*P*= 0.04) in experiment one (Table 2). In the second experiment, there was no significant difference between prokaryotic rhizosphere communities of infected and uninfected plants.

3.5.3.2 No significant endophyte-associated impacts on the fungal rhizosphere microbiome

PCoA plots of fungal rhizosphere community composition showed that in the first experiment rhizosphere samples of infected and uninfected plants clearly clustered together. In the second experiment there was apparent partitioning of infected and uninfected rhizosphere samples (Figure 17 b), however the difference was not statistically significant (Table 2). This suggests that endophyte infection did not significantly affect overall community composition of fungal rhizosphere communities in either experiment, although there was an apparent but non-significant difference of communities in the rhizosphere of infected versus uninfected plants in the second experiment but not the first- the significance of which may have been demonstrable with a larger sample size.

3.5.3.3 Significant differentiation of prokaryotic and fungal communities depending on sample type and experiment

Consistent with the results of alpha-diversity analyses, significant differentiation between bulk soil and rhizosphere communities were observed in both experiments (Table 2). Regardless of sample type, for both prokaryotes and fungi there was also highly significant ($P \leq 0.003$) partitioning of samples between each experiment, both between sample types of each experiment (Figure 17 a, b) as well as between all samples of each experiment irrespective of sample type (Figure 17 c, d).

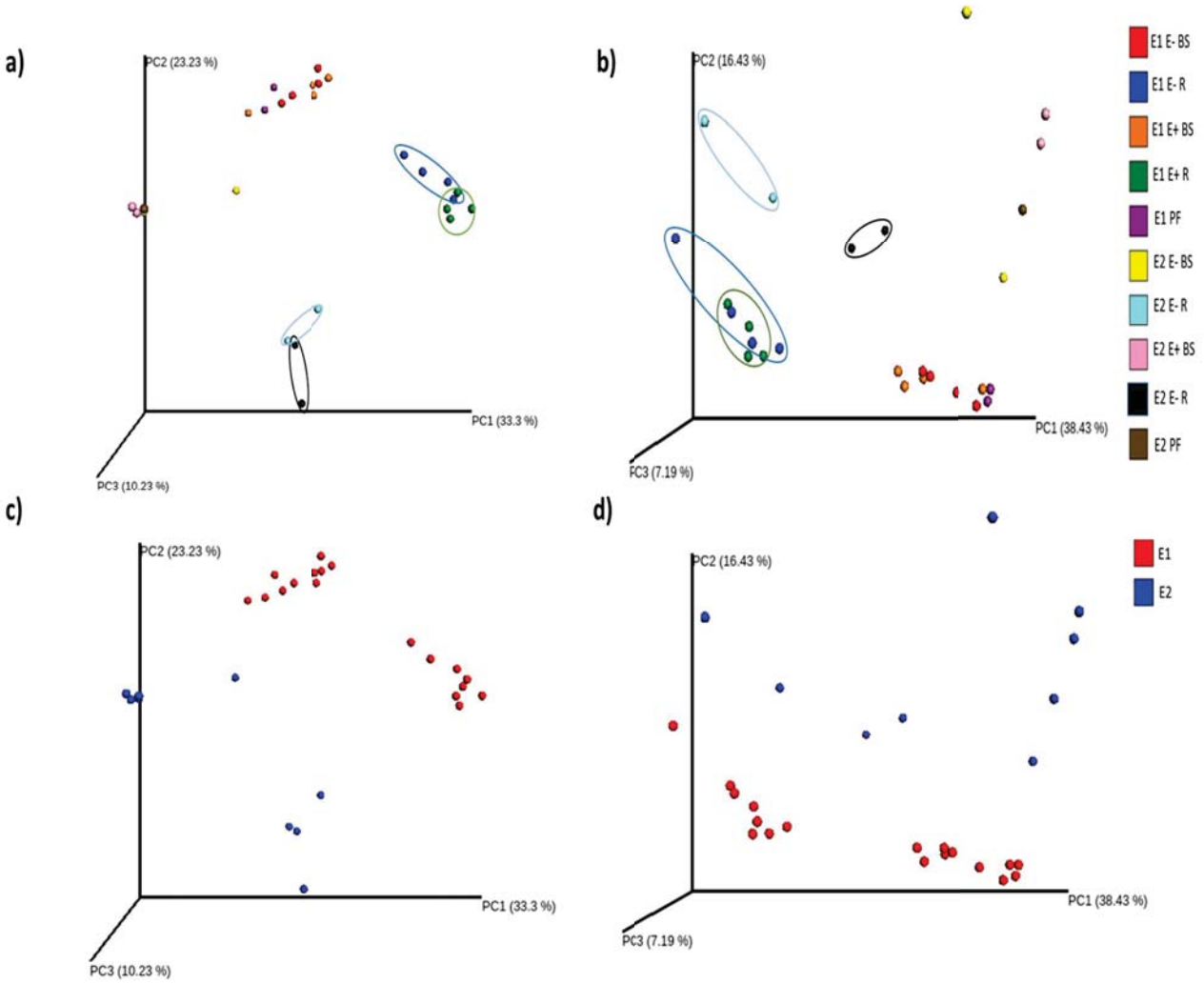


Figure 17. Principal coordinates analysis plots of Bray-Curtis dissimilarity matrices. Plots of Prokaryotic (a, c) and fungal (b, d) samples grouped by experiment, sample type and endophyte treatment (a, b), and by only experiment (c, d). Percentages on each axis represents the percentage of variation explained along that particular axis. E1/E2= Experiment one/two, PF= Plant-free, BS= Bulk-soil, R= Rhizosphere, E+/- = endophyte-infected/uninfected.

Table 2. PERMANOVA comparisons of beta-diversity between sample groupings.

Taxon	Comparison[1]	Pseudo-F	P-value[2]
Prokaryotes	**E1 vs E2**	**8.9**	**0.001**
	E1 BS vs E2 BS	**7.2**	**0.001**
	E1 R vs E2 R	**11.7**	**0.003**
	E1 BS vs E1 R	**12.0**	**0.001**
	E1 E+ BS vs E1 E- BS	0.77	0.73
	E1 E+ R vs E1 E- R	**1.25**	**0.04**
	E2 BS vs E2 R	**2.7**	**0.03**
	E2 E+ BS vs E2 E- BS	1.0	0.66
	E2 E+R vs E2 E- R	0.97	0.67
Fungi	**E1 vs E2**	**4.7**	**0.002**
	E1 BS vs E2 BS	**5.5**	**0.003**
	E1 R vs E2 R	**4.4**	**0.002**
	E1 BS vs E1 R	**12.8**	**0.002**
	E1 E+ BS vs E1 E- BS	0.8	0.75
	E1 E+ R vs E1 E- R	1.1	0.27
	E2 BS vs E2 R	**4.3**	**0.03**
	E2 E+ BS vs E2 E- BS	0.77	1.0
	E2 E+ R vs E2 E- R	1.9	0.3

[1] E1/E2= Experiment one/two, BS= Bulk-Soil, R= Rhizosphere, E+/-= Endophyte-infected/uninfected.

[2] False discovery rate (FDR)-corrected. Significant ($P \leq 0.05$) comparisons are highlighted in bold. The only significant comparison between rhizospheres of infected and uninfected rhizosphere samples is highlighted in red.

In summary, results of beta-diversity analyses showed that aside from a subtle yet significant shift in prokaryotic community composition in the first experiment but not the second, endophyte infection had no significant impact on overall rhizosphere community composition of their hosts. In contrast, similarly to the findings of alpha-diversity analyses, both prokaryotic and fungal rhizosphere communities of plants from each experiment significantly differed irrespective of endophyte infection, as well as between bulk soil and rhizosphere samples within each experiment.

3.5.4 Taxonomic analyses

While alpha and beta-diversity analyses allowed insights into the relative differences in levels of diversity within samples as well as overall community composition between samples, neither of which provided any indication as to which specific taxonomic groups differed between samples. To assess this, relative abundance taxa plots were generated.

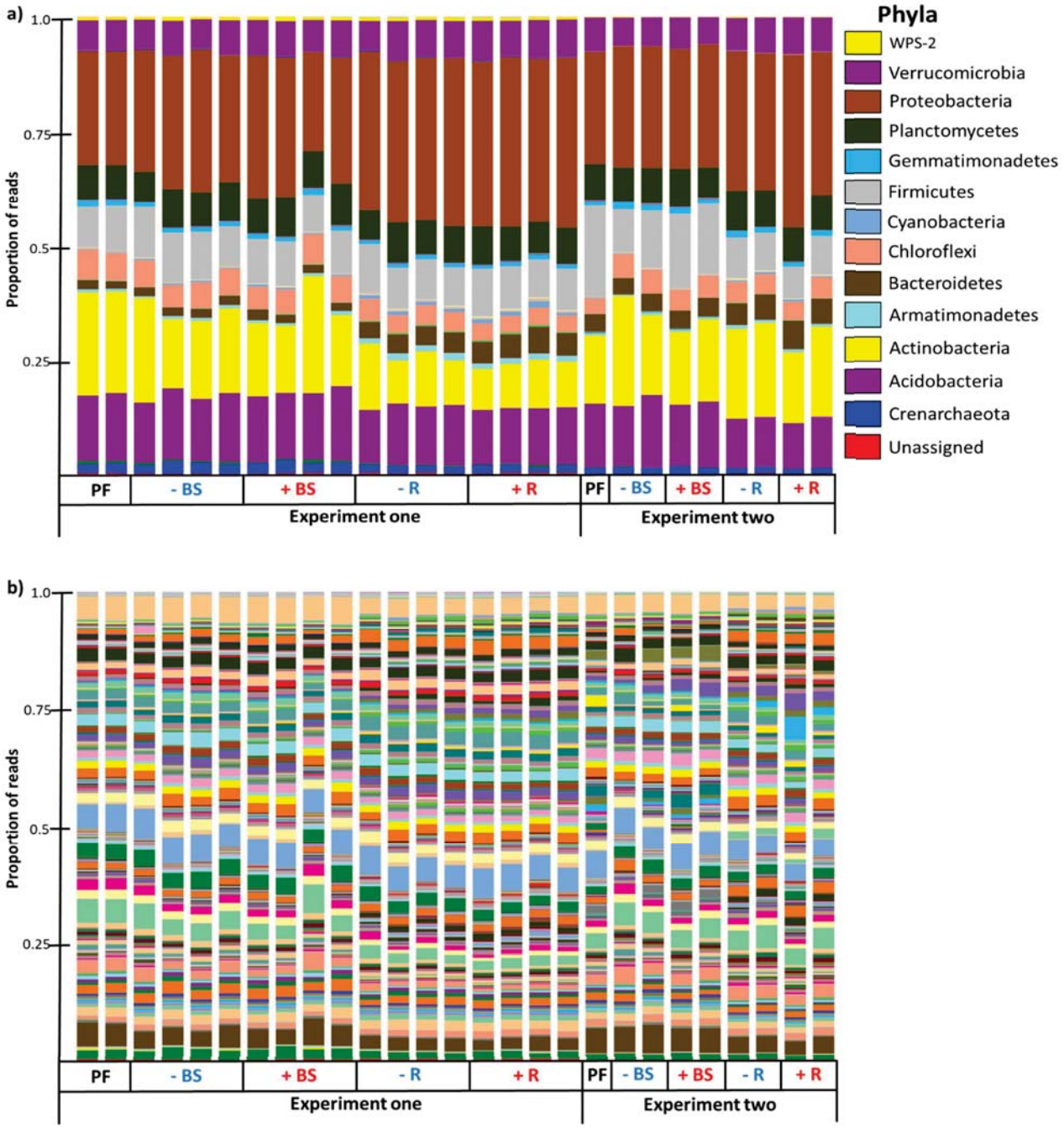
Taxa plots of community composition in each replicate are shown at both the level of phyla (Figure 18 a, c), as well as the genus level (Figure 18 b, d). Additionally, differential abundances of individual OTUs between rhizospheres of infected and uninfected plants was assessed using DESeq2 (Love et al., 2014)- this firstly normalizes the data by fitting it to a negative binomial distribution, followed by Bayesian shrinkage to scale log fold change towards zero. The statistical significance of differential abundances of OTU counts between treatments is then calculated using a Wald test (Love et al., 2014). Based on the results of beta-diversity analyses, it was hypothesized that differences in community composition between rhizosphere microbiomes of infected and uninfected plants would be minor in magnitude compared to differences between rhizospheres of plants of each experiment irrespective of endophyte status, as well as between bulk soil and rhizosphere communities within experiments. However, the apparent but non-significant partitioning of fungal rhizosphere samples from infected and uninfected plants observed in the second experiment suggested that there could be greater differences in fungal rhizosphere community composition in this experiment compared with the first.

3.5.4.1 Minor impact of endophyte infection on prokaryotic rhizosphere community composition

In both experiments, there was only slight variability in prokaryotic community composition of the rhizosphere of infected versus uninfected plants- both at the level of phyla (Figure 18 a) as well as the genus level (Figure 18 b). In experiment one, the only statistically significant difference between endophyte treatments was a 1.1% increase in the relative abundance of Bacteroidetes in the rhizosphere of infected compared with uninfected plants (Table 3). At the genus level, in both experiments taxa profiles of rhizosphere communities of infected and uninfected plants displayed a high degree of similarity (Figure 18 b).

At the level of OTUs, in the first experiment only one differentially abundant OTU assigned as belonging to the Actinobacterial family Patulibacteraceae (Albuquerque & da Costa, 2014) was present in significantly lower relative abundances in the rhizosphere of infected versus uninfected plants. Nine significantly differentially abundant OTUs were identified in

the second experiment, although all of which were present in very low relative abundances (<0.3%) (Table 4). Rather than being of differential abundance in each treatment, all identified differentially abundant prokaryotic OTUs of the second experiment were absent from one of the two treatments.


a)
b)
1.0
0.75
0.5
0.25
Proportion of reads
Phyla
WPS-2
Verrucomicrobia
Proteobacteria
Planctomycetes
Gemmatimonadetes
Firmicutes
Cyanobacteria
Chloroflexi
Bacteroidetes
Armatimonadetes
Actinobacteria
Acidobacteria
Crenarchaeota
Unassigned
PF
- BS
+ BS
- R
+ R
Experiment one
Experiment two

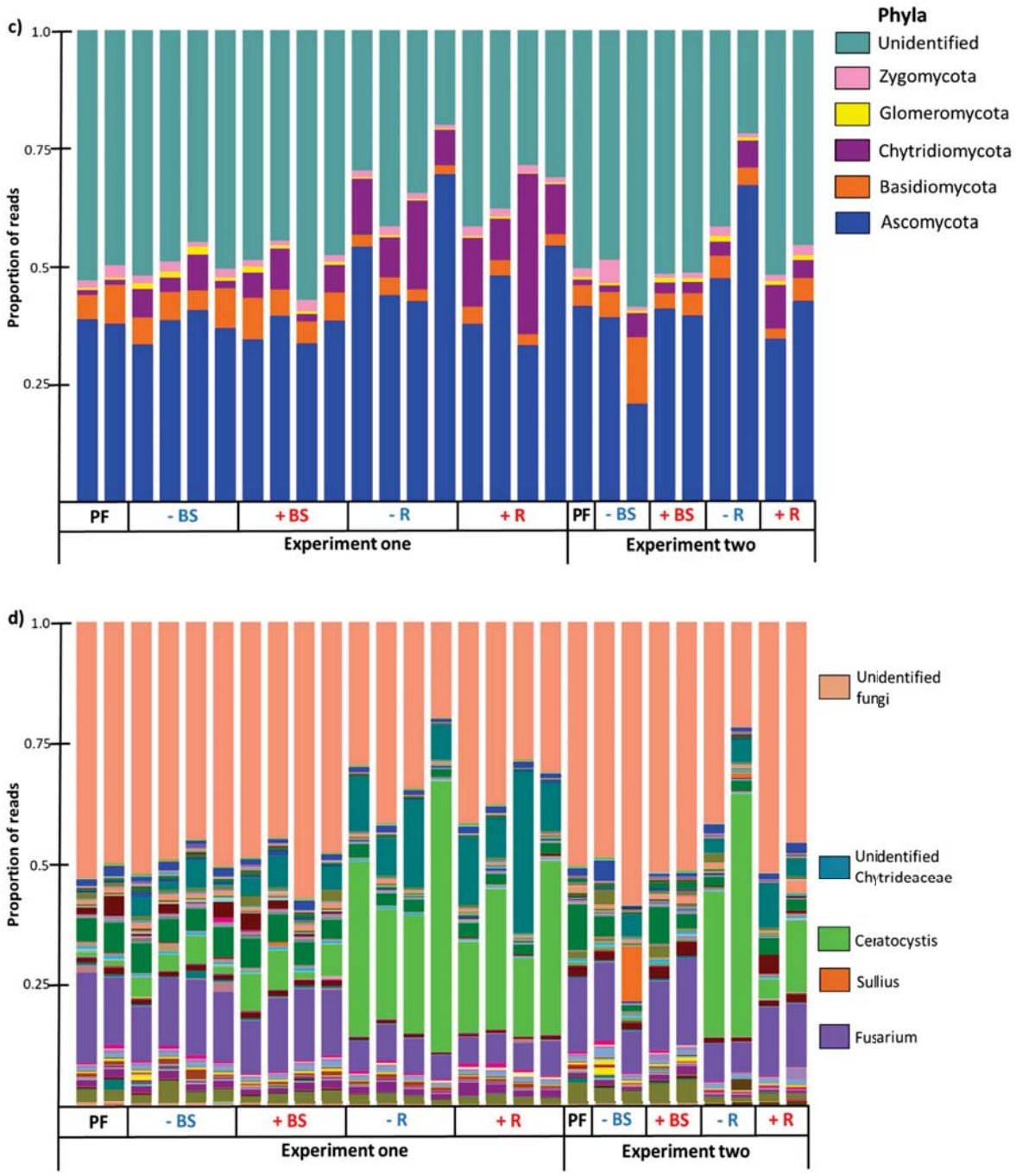


Figure 18. Relative abundance taxa plots of individual samples. Both prokaryotic and fungal samples shown at both the level of phyla (a, c) and genus (b, d). In all categories, taxa plots of replicates are placed in numerical order. Phyla present in overall relative abundances less than 0.2% were omitted from the legend in a), however the full legend is shown in appendix A7. The legend in a) is ordered in the same order as taxa appear on the plots. Due to their very large size full legends for b) and d) are not shown, as aside from labelled fungi in d) these are instead only intended to act as visual indicators of similarity/dissimilarity between samples. BS= Bulk-soil; R= Rhizosphere. Blue/red font= endophyte-infected/uninfected

3.5.4.2 Endophyte-associated effects towards rhizosphere fungi largely limited to reductions in abundances of a single OTU likely of the class Sordariomycetes in both experiments

Relative abundance taxa plots of fungal communities in each individual sample at the level of phyla as well as the genus level are shown in Figure 18 c and d, respectively. In the first experiment, there were apparent decreases in the relative abundances of Ascomycota which coincided with increased abundances of Chytridiomycota in the rhizospheres of infected compared with uninfected plants, although none of these differences were statistically significant (Table 3). At the genus level, in the first experiment there was an apparent decrease in the relative abundances of *Ceratocystis* (de Beer et al.,2014) in the rhizosphere of infected versus uninfected plants in both experiments. Strikingly, filtering the OTU table of OTUs that were assigned as belonging to the genus *Ceratocystis* found that an average of 98% of total *Ceratocystis* reads in rhizosphere samples mapped to a single OTU (Figure 19 a). Despite this trend being apparent in both experiments (Figure 19 b), its significance could not be demonstrated within either experiment (Table 4). To test if endophyte infection was associated with a lower abundance of this OTU across both experiments, the percentages of all reads in each of the 12 rhizosphere samples associated with the '*Ceratocystis*' OTU were log-transformed and then regressed simultaneously against both experiment (1 or 2) and infection status (positive or negative), to adjust for variability between experiments. The analysis demonstrated that endophyte infection did significantly lower relative abundances of this OTU (one-sided $P = 0.0264$).

To better ascertain the confidence with which this identified OTU was assigned taxonomy, the representative sequence of this OTU was run through Basic Local Alignment Search tool (BLAST) (Altschul et al., 1997) searches manually using the UNITE (Kojalg et al., 2013) and 'nr' BLAST databases. Unexpectedly, while this sequence mapped with 94-96% similarity to three plant-pathogenic *Ceratocystis* species (*C. paradoxa*, *C. ethacetica*, and *C. radicola*), this alignment only occurred across the first 90 nucleotides at the 5' end of the entire 266 nucleotide representative OTU sequence. However, the entire OTU sequence aligned with 78-90% similarity to a number of deposited sequences assigned as belonging to species within the class Sordariomycetes (Appendix A8). This shows that despite the OTU being assigned to the genus *Ceratocystis* during taxonomy assignment in QIIME, given that

this was only based on an alignment of the first 90/266 nucleotides at the 5' end of the reference sequence the identity of this OTU at lower taxonomic levels is largely uncertain. However, the moderate homology of this sequence to a number of deposited sequences assigned as belonging to Sordariomycetes suggests that the species represented by this OTU is likely of this class.

Aside from this observed endophyte-associated reduction in the relative abundances of this OTU, effects of endophyte infection towards fungal rhizosphere community composition were otherwise minor. In experiment two there was also an apparent but non-significant endophyte-associated increase in the relative abundances of *Fusarium* (6.9 ± 1.1% in uninfected versus 13.6 ± 0.7% in infected plants), although not in the first experiment (6.4 ± 1.1 in uninfected versus 6 ± 0.8% in infected plants, (*P*= 0.44)). Three significantly differentially abundant fungal OTUs were identified in experiment two, although the only one which could be assigned taxonomy belonged to the Basidiomycete genus *Sullius* (Table 3). However, in Figure 18 c it is clearly seen that *Sullius* was present in a much higher relative abundance in the bulk soil of the second endophyte-negative replicate (11.3% versus < 0.1% in the other three replicates). As a result, it is likely that the differential abundance of this OTU was due to this disproportionately high relative abundance of this genus in the bulk soil of this single replicate rather than being due to endophyte infection. As for prokaryotes, all identified significantly differentially abundant fungal OTUs of the second experiment were absent from one of the two treatments as opposed to being of differential abundance in both treatments.

Overall, the main effect identified between rhizospheres of infected and uninfected plants was the reduction of a single OTU which accounted for an average of 98% of all total *Ceratocystis* reads in rhizosphere samples (Figure 19 a) of infected compared with uninfected plants in both experiments. While the significance of this effect could not be demonstrated in individual experiments (Table 4), fitting the data from both experiments to a linear regression model which controlled for variability between experiments showed that the endophyte-associated reduction in abundances of this OTU were statistically significant (19.2 ± 11.1% in infected versus 35.9 ± 13.6% in uninfected; *P*(one-sided)= *0.0264*). Manual BLAST searches of the representative sequence of this OTU using the UNITE and 'nr' BLAST databases revealed that only the first 90 bases at the 5' end of the

OTU sequence aligned with ITS sequences assigned as belonging to the *Ceratocystis* species *C. paradoxa*, *C. ethacetica*, and *C. radicola*. However, >95% of the entire representative OTU sequence mapped with 76-90% similarity to a number of reference sequences assigned as belonging to the class Sordariomycetes, suggesting that the species represented by this OTU is likely of this class. Aside from this effect towards this abundant OTU, other endophyte associated effects towards rhizosphere fungi were minor in magnitude and only occurred in one of the two experiments of this study.

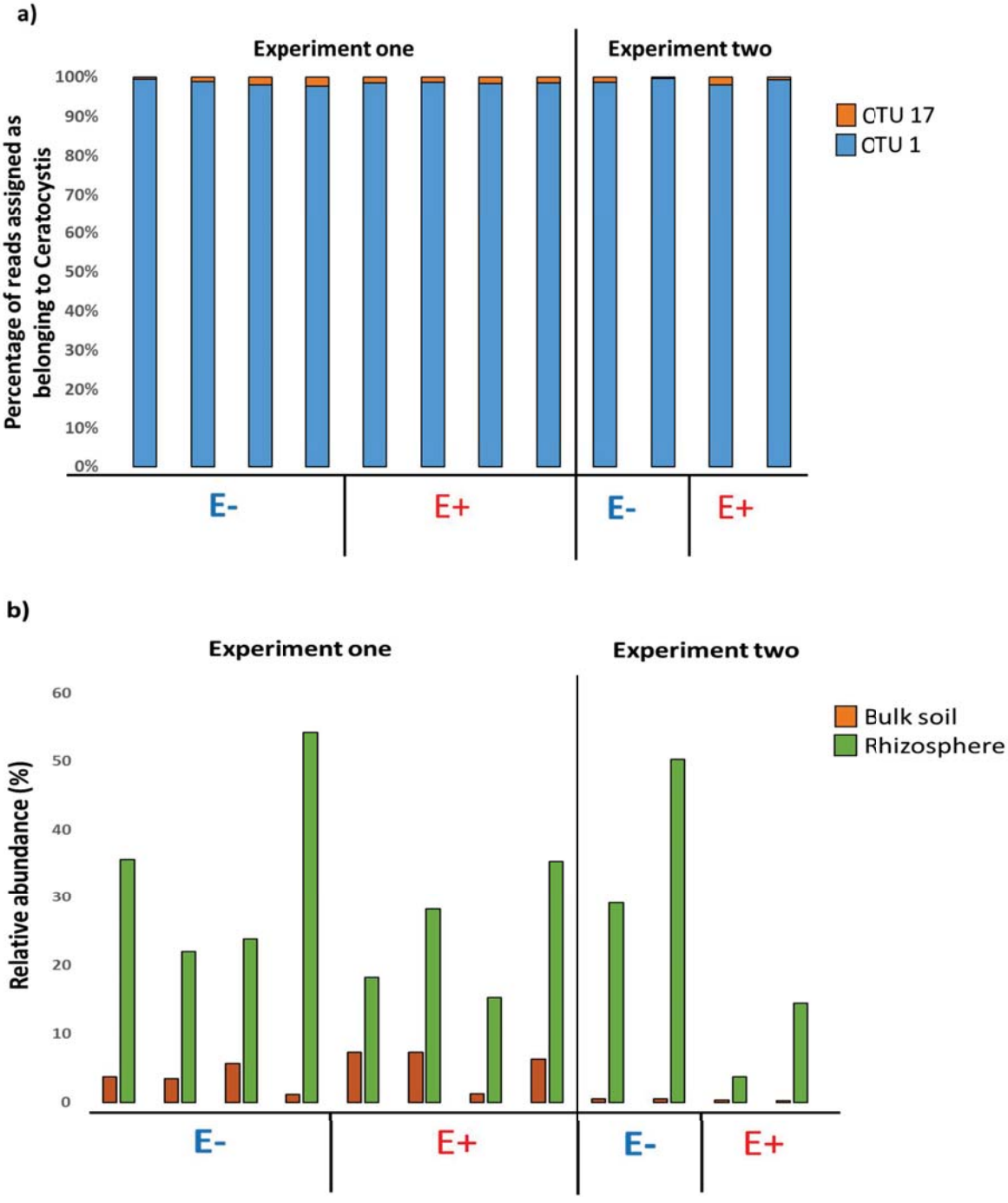


Figure 19. Reduced abundances of OTU 1 in the rhizosphere of infected versus uninfected plants. **a)** Stacked bar graphs showing the overall proportion of reads mapping to the genus *Ceratocystis* which corresponded to OTU 1 and OTU 17 in the unrarefied dataset. **b)** Bar graph showing the percentages of OTU 1 reads in bulk-soil (brown bars) versus rhizosphere (green bars) samples for each replicate. In each category of both graphs, replicates are shown in numerical order. E+/-= Endophyte infected/uninfected.

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Table 3. Comparisons of relative abundances of prokaryotic and fungal phyla between rhizosphere samples of infected and uninfected plants.

Experiment	Taxon	Phylum	Mean E+R relative abundance	Mean E-R relative abundance	P-value[1,2]
1	Prokaryotes	Proteobacteria	36.3 ± 0.8	35.3 ± 0.9	0.1
		Actinobacteria	9.7 ± 0.7	11.4 ± 2.4	0.24
		Bacteroidetes	**5.2 ± 0.5**	**4.1 ± 0.4**	**0.014**
		Chloroflexi	3.7 ± 0.1	4.2 ± 0.3	>0.5
		Acidobacteria	12.5 ± 0.3	13 ± 0.8	0.44
		Crenarchaeota	1.8 ± 0.2	1.7 ± 0.1	>0.5
		Planctomycetes	7.7 ± 0.6	7.7 ± 1	>0.5
	Fungi	Firmicutes	9.3 ± 0.8	9.3 ± 1.1	0.44
		Ascomycota	43.3 ± 9.6	52.5 ± 12.5	0.24
		Basidiomycota	3 ± 0.7	2.7 ± 0.8	>0.5
		Chytridiomycota	17 ± 11.6	11.8 ± 5.2	0.24
		Zygomycota	1.7 ± 0.4	1.4 ± 0.5	0.44
		Unidentified	34.8 ± 6	31.5 ± 9.1	0.34
2	Prokaryotes	Proteobacteria	34.2 ± 4.5	29.9 ± 0.2	n/a
		Actinobacteria	17.8 ± 2.9	20.3 ± 0.6	n/a
		Bacteroidetes	5.9 ± 0.6	5 ± 0.6	n/a
		Chloroflexi	4.2 ± 0.4	4.4 ± 0.1	n/a
		Acidobacteria	10.5 ± 0.8	10.7 ± 0.1	n/a
		Crenarchaeota	1.1 ± 0.1	1.4 ± 0.1	n/a
		Planctomycetes	7.6 ± 0.1	8.4 ± 0.6	n/a
	Fungi	Firmicutes	7.7 ± 1.1	8.6 ± 0.4	n/a
		Ascomycota	38.6 ± 5.6	57.3 ± 13.9	n/a
		Basidiomycota	3.5 ± 1.9	4.3 ± 0.8	n/a
		Chytridiomycota	6.6 ± 3.8	4.4 ± 2	n/a
		Zygomycota	1.8 ± 0.6	1.5 ± 0.8	n/a
		Unidentified	48.8 ± 4.5	31.8 ± 14.1	n/a

[1] Significance was assessed using the Mann-Whitney U-test (two-tailed, P≤0.05). Significant comparisons are highlighted in bold.

[2] Due to only two replicates per treatment analysed in the second experiment, statistical analysis comparing rhizospheres of differing endophyte status using the Mann-Whitney U test could not be assessed.

Table 4. Significantly differentially abundant prokaryotic OTUs between rhizospheres of infected and uninfected plants.

Experiment	Taxon	OTU id	Predicted taxonomy[1]	Mean E+ relative abundance (%)[2]	Mean E- relative abundance (%)[2]	P-value[3]
1	**Prokaryotes**	76	Actinobacteria f; Patulibacteracae	0.15 ± 0.01	0.27 ± 0.09	0.01
	Fungi	107	Unidentified	0	0.15 ± 0.07	0.009
		19	Unidentified	1.75 ± 1.1	0.67 ± 0.5	0.02
		1	**Ascomycota g; *Ceratocystis***	**23.1 ± 6**	**36.5 ± 20.6**	**0.35**
2	**Prokaryotes**	3579	Proteobacteria f; Comamonadaceae	0.22 ± 0.09	0	0.001
		294	Proteobacteria f; Phyllobacteriaceae	0.18 ± 0.07	0	0.005
		242	Actinobacteria f; Micromonosporaceae	0	0.13 ± 0.02	0.01
		417	Proteobacteria f; Pseudomonas	0.16 ± 0.07	0	0.01
		183	Planctomycetes f; WD2101	0	0.13 ± 0.01	0.01
		408	Proteobacteria f; Methylocystaceae	0	0.11 ± 0.01	0.01
		246	Firmicutes; f_Planococcaceae	0	0.11 ± 0.01	0.01
		1021	Proteobacteria; f_Comamonadaceae	0.12 ± 0.01	0	0.01
		687	Actinobacteria; o_Actinomycetales	0.11 ± 0.003	0	0.02
	Fungi	29	Agaricomycetes; g *Suillus.*	0	0.52 ± 0.6	$1.2x10^{-6}$
		115	Unidentified	0	0.45 ± 0.46	$1.1x10^{-5}$
		91	Unidentified	0.24 ± 0.2	0	0.04
		1	**Ascomycota; g *Ceratocystis***	**9.1 ± 7.5**	**39.7 ± 14.9**	**0.053**
		2	**Ascomycota; g *Fusarium***	**6.5 ± 1.4**	**12.7 ± 1.8**	**>0.5**

[1] Phyla followed by the highest level of taxonomy classified to each OTU up to the genus level (o= order, f= family, g= genus).

[2] Abundances were calculated as the average proportion of reads per unrarefied sample corresponding to that OTU divided by the total number of reads from that sample.

[3] False discovery rate (FDR)-corrected *P*-value generated by DESeq2. OTUs which showed apparent but non-significant differential abundances between endophyte treatments are highlighted in bold.

3.5.4.3 Comparatively larger differences in prokaryotic and fungal rhizosphere community composition between experiments than between rhizospheres of plants of differing endophyte status within each experiment

Results of both alpha and beta-diversity analyses suggested that rhizosphere communities of plants differed to a greater extent between plants of each experiment than between infected and uninfected plants within experiments. To assess this, taxa plots were generated where samples were collapsed into single plots depending on their endophyte status within experiments versus between experiments irrespective of endophyte infection (Figure 20). At the level of phyla, the only significant differences identified between experiments were a 2.1% decrease in Acidobacteria and an 8.4% increase in Actinobacteria in the second experiment compared with the first (Table 5). At the genus level, a slightly greater shift in community composition was seen between rhizospheres depending on experiment rather than endophyte infection in each experiment, although this difference was minimal (Figure 20 b). At the level of OTUs, a total of 112 prokaryotic OTUs were significantly differentially abundant between experiments (Appendix A9)- far more than were found to be differentially abundant within each experiment between endophyte treatments (Table 4).

For fungi, at the level of phyla the only statistically significant difference in rhizosphere community composition between experiments was an 8.9% decrease in the relative abundance of Chytridiomycota in the second experiment compared with the first. There were also increases in the relative abundances of Glomeromycota and unidentified fungi in the second experiment compared to the first, however these differences were non-significant (Table 5). In comparison to the variability seen in rhizospheres of infected and uninfected plants, there was a similar degree of variability in fungal rhizosphere community composition of plants between experiments as there were due to endophyte infection in each experiment (Figure 20 c, d). For example, the differences in relative abundances of Ceratocystis between infected and uninfected plants within experiments was greater than between experiments irrespective of endophyte infection (Figure 20 c). However, it is important to note that fungal rhizosphere communities displayed a high degree of variability between replicates within treatments (Figure 18 c, d) in the first experiment, and in the second experiment while these effects were consistent they were based on only two

biological replicates per treatment. At the level of OTUs, a total of 45 fungal OTUs were significantly differentially abundant between rhizospheres of each experiment (Appendix A9)- far more than were identified between rhizospheres of infected and uninfected plants within each experiment (Table 4).

Overall, these results suggest that the greater extent of differences in both prokaryotic and fungal rhizosphere community composition between experiments irrespective of endophyte infection compared with between samples of differing endophyte status within each experiment were largely due to a greater extent of differences at the level of OTUs.

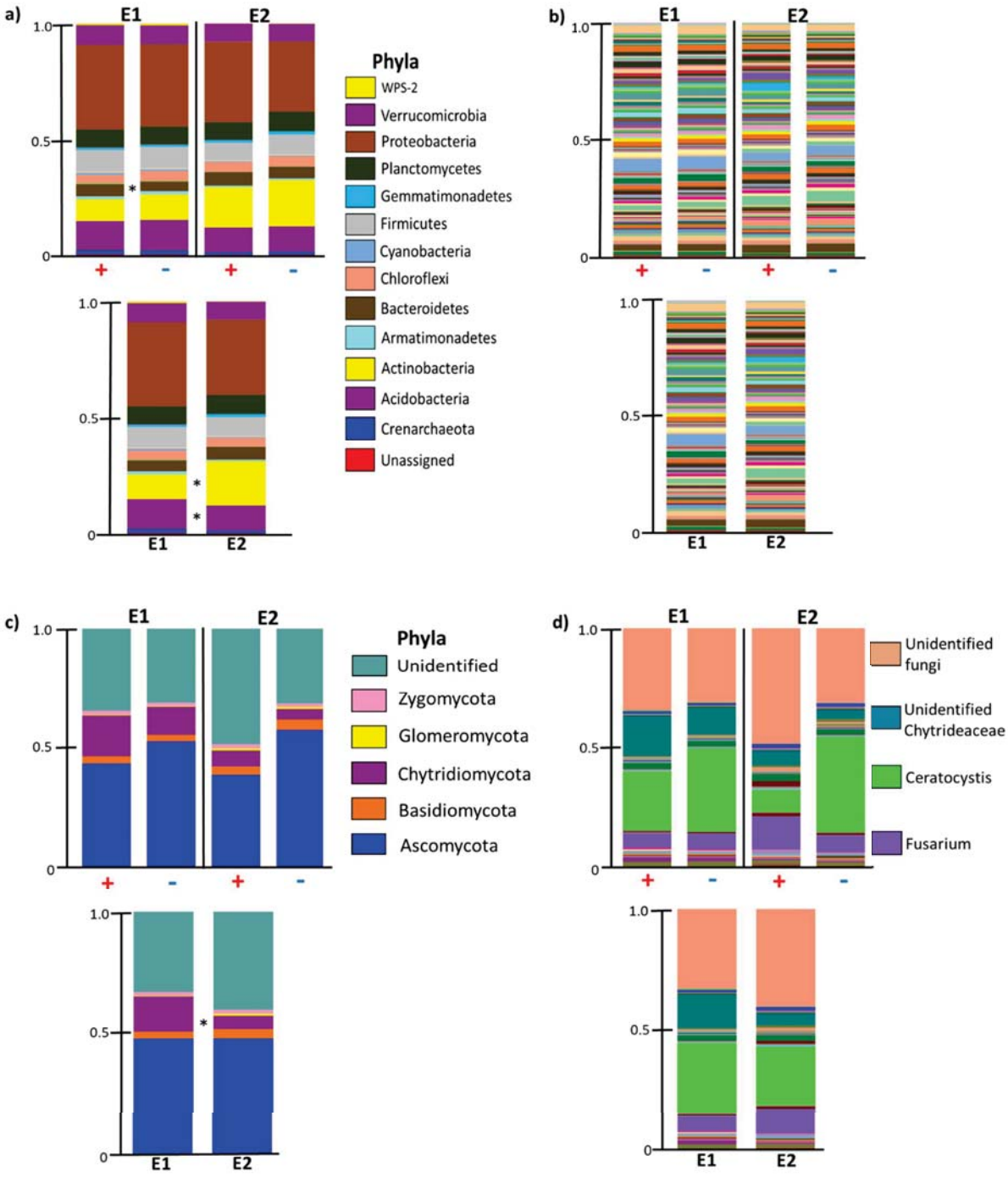

Figure 20. Comparison of rhizosphere samples based on endophyte status within experiments versus between experiments irrespective of endophyte infection. Prokaryotes (a, b) and fungi (c, d), at the level of phyla (a, c) and at the genus level (b, d). Taxa plots represent averages of all infected (red crosses) and uninfected (blue dashes) rhizosphere samples within each experiment, as well as plots representing all rhizosphere samples from each experiment irrespective of endophyte infection (E1/E2= Experiment one/two). Significantly differentially abundant phyla in each comparison contain an asterisk next to them.

Table 5. Between-experiment comparisons of relative abundances of prokaryotic and fungal phyla.

Comparison[1]	Taxon	phylum	Mean E1 relative abundance (%)	Mean E2 relative abundance (%)	P-value of difference[2]
E1R vs E2R	**Prokaryotes**	Proteobacteria	35.8 ± 0.9	32.1 ± 3.6	0.1
		Acidobacteria	**12.7 ± 0.6**	**10.6 ± 0.5**	**0.024**
		Actinobacteria	**10.6 ± 1.8**	**19 ± 2.2**	**0.008**
		Firmicutes	9.3 ± 0.9	8.1 ± 0.9	>0.5
		Bacteroidetes	4.6 ± 0.7	5.4 ± 0.7	0.1
		Planctomycetes	7.7 ± 0.8	8 ± 0.6	>0.5
		Gemmatimonadetes	0.8 ± 0.1	1.2 ± 0.2	>0.5
		Verrucomicrobia	8 ± 0.7	7.6 ± 0.4	>0.5
		Chloroflexi	3.9 ± 0.4	4.3 ± 0.3	>0.5
		Crenarchaeota	1.7 ± 0.2	1.2 ± 0.2	>0.5
	Fungi	Ascomycota	47.9 ± 11.4	47.9 ± 13.8	>0.5
		Basidiomycota	2.8 ± 0.7	3.9 ± 1.3	>0.5
		Chytridiomycota	**14.4 ± 8.8**	**5.5 ± 2.8**	**0.014**
		Glomeromycota	0.3 ± 0.1	0.9 ± 0.2	0.3
		Zygomycota	1.5 ± 0.4	1.6 ± 0.6	>0.5
		Unidentified	33.1 ± 7.3	40.3 ± 13	0.18
E1 BS vs E2 BS	**Prokaryotes**	Proteobacteria	27.9 ± 0.9	26.3 ± 3.6	0.3
		Actinobacteria	18 ± 1.8	18.7 ± 2.2	0.2
		Firmicutes	9.25 ± 0.9	13.4 ± 0.9	0.1
		Bacteroidetes	1.9 ± 0.7	3.8 ± 0.7	0.4
		Planctomycetes	8.1 ± 0.8	7.6 ± 0.7	>0.5
		Gemmatimonadetes	1 ± 0.2	1.3 ± 0.3	>0.5
		Verrucomicrobia	7.3 ± 0.6	6.3 ± 0.5	0.18
		Chloroflexi	5.5 ± 0.4	5 ± 0.3	>0.5
		Crenarchaeota	2.5 ± 0.2	1.6 ± 0.2	>0.5
	Fungi	Ascomycota	36.9 ± 2.8	35 ± 9.5	0.3
		Basidiomycota	6.1 ± 1.6	6.9 ± 4.9	0.4
		Chytridiomycota	5 ± 2.5	2.8 ± 1.5	0.3
		Glomeromycota	1 ± 0.4	0.7 ± 0.2	>0.5
		Zygomycota	1.6 ± 0.5	2 ± 2	>0.5
		Unidentified	49.4 ± 3.8	52.7 ± 4.3	0.14
All E1 vs all E2	**Prokaryotes**	Proteobacteria	31 ± 4.7	26.3 ± 4	0.2
		Acidobacteria	14.8 ± 1.7	14.4 ± 2.1	0.1
		Actinobacteria	15.1 ± 4.8	18.7 ± 2.9	0.07
		Firmicutes	9.5 ± 2.8	13.4 ± 4.5	>0.5
		Bacteroidetes	**3.1 ± 1.5**	**3.8 ± 1**	**0.04**
		Planctomycetes	7.9 ± 0.7	7.8 ± 0.6	>0.5

	Gemmatimonadetes	**1 ± 0.3**	**1.2 ± 0.2**	**0.008**
	Verrucomicrobia	7.5 ± 0.7	7 ± 0.8	0.08
	Chloroflexi	4.9 ± 0.9	5 ± 0.6	0.4
	Crenarchaeota	**2.1 ± 0.5**	**1.4 ± 0.3**	**0.0007**
Fungi	Ascomycota	41.9 ± 9.3	41.5 ± 12.1	0.5
	Basidiomycota	4.7 ± 2.2	5.2 ± 3.5	>0.5
	Chytridiomycota	8.7 ± 7.9	3.8 ± 2.6	0.07
	Glomeromycota	0.6 ± 0.4	0.7 ± 0.2	0.1
	Zygomycota	1.6 ± 0.5	1.8 ± 1.3	>0.5
	Unidentified	42.4 ± 10.1	46.9 ±10.5	0.2

[1] E1R vs E2R= All rhizosphere samples of experiment one versus all rhizosphere samples from experiment two.

E1BS vs E2BS= All bulk soil samples of experiment one versus all rhizosphere samples from experiment two (excluding samples from plant-free controls).

All E1 vs All E2= All samples of experiment one versus all samples from experiment two, including samples from plant-free controls.

[2] Significance was assessed using the Mann-Whitney U-test (two-tailed, $P \leq 0.05$). Significant comparisons are highlighted in bold.

3.5.4.4 Significant differences in relative abundances of a range of prokaryotic and fungal phyla between bulk soil and rhizosphere samples within each experiment

Irrespective of endophyte status, a number of prokaryotic and fungal phyla significantly differed between bulk soil and rhizosphere samples in both experiments (Table 6). For prokaryotes, in both experiments rhizospheres had significantly higher relative abundances of Proteobacteria and Bacteroidetes relative to bulk soil, whereas relative abundances of Acidobacteria, Chloroflexi and Crenarchaeota were instead significantly enriched in bulk soil compared with the rhizosphere (Table 6). Actinobacteria were also significantly enriched in the bulk soil, although only in the first experiment. Aside from Actinobacteria all other prokaryotic phyla shown the same trend of enrichment between rhizosphere and bulk soil communities in the second experiment, however the significance of differences in Planctomycetes, Gemmatimonadetes, Chloroflexi and Crenarchaeota were not demonstrable. Aside from Glomeromycota the same general trends of enrichment between bulk soil and rhizosphere samples were also shown in the relative abundances of fungal phyla between sample types, however in experiment two none of the differences were significant (Table 6). Interestingly, the OTU identified as being

differentially abundant in the rhizosphere of infected versus uninfected plants was also highly enriched in the rhizosphere compared with bulk soil in both experiments (E1, 5.6 ± 3.1% in BS versus 34.4 ± 14.1% in R; E2, 0.5 ± 0.2% in BS versus 28.2 ± 23% in R) (see Figure 19 b), suggesting that the taxa represented by this OTU are well-equipped to flourish in the rhizosphere of perennial ryegrass despite only being present in much lower relative abundances in the wider bulk soil.

Table 6. Within-experiment comparisons of prokaryotic and fungal phyla between bulk soil and rhizosphere samples.

Experiment	Taxon	Phylum	Mean BS relative abundance (%)[1]	Mean R relative abundance (%)[1]	P-value of difference[2]
1	**Prokaryotes**	**Proteobacteria**	**27.9 ± 3.2**	**35.8 ± 1**	**0.0009**
		Actinobacteria	**18 ± 3.9**	**10.5 ± 1.8**	**0.001**
		Acidobacteria	**14.8 ± 1**	**12.7 ± 0.6**	**0.007**
		Bacteroidetes	**1.9 ± 0.2**	**4.6 ± 0.7**	**0.0009**
		Firmicutes	9.7 ± 1	9.3 ± 0.9	0.29
		Planctomycetes	8.1 ± 0.8	7.7 ± 0.8	0.29
		Gemmatimonadetes	**1 ± 0.2**	**0.8 ± 0.1**	**0.03**
		Verrucomicrobia	**7.3 ± 0.6**	**8 ± 0.7**	**0.02**
		Chloroflexi	**5.5 ± 0.6**	**3.9 ± 0.4**	**0.0009**
		Crenarchaeota	**2.5 ± 0.1**	**1.7 ± 0.2**	**0.002**
	Fungi	**Ascomycota**	**36.9 ± 2.8**	**47.9 ± 11.4**	**0.04**
		Chytridiomycota	**5 ± 2.5**	**14.4 ± 8.8**	**0.0028**
		Basidiomycota	**6.1 ± 1.6**	**2.8 ± 0.7**	**0.0009**
		Zygomycota	1.6 ± 0.5	1.5 ± 0.4	0.4
		Glomeromycota	**1 ± 0.4**	**0.3 ± 0.1**	**0.0009**
		Unidentified	**49.4 ±3.8**	**33.1 ±7.3**	**0.0009**
	Prokaryotes	**Proteobacteria**	**26.3 ± 0.3**	**32.1 ± 3.6**	**0.01**
2		Actinobacteria	18.7 ± 3.4	19 ± 2.2	>0.5
		Acidobacteria	**14.4 ± 1.3**	**10.6 ± 0.5**	**0.01**
		Bacteroidetes	**3.8 ± 0.1**	**5.4 ± 0.7**	**0.01**
		Firmicutes	**13.4 ± 3.1**	**8.1 ± 0.9**	**0.01**
		Planctomycetes	7.6 ± 0.7	7.6 ± 0.4	>0.5
		Gemmatimonadetes	1.3 ± 0.3	1.2 ± 0.2	>0.5
		Verrucomicrobia	**6.3 ± 0.5**	**7.6 ± 0.4**	**0.01**
		Chloroflexi	5 ± 0.3	4.3 ± 0.3	>0.5
		Crenarchaeota	1.6 ± 0.2	1.2 ± 0.2	0.17
	Fungi	Ascomycota	35 ± 9.5	47.9 ± 13.8	0.1
		Chytridiomycota	2.8 ± 1.5	5.5 ± 2.8	0.34
		Basidiomycota	6.9 ± 4.9	3.9 ± 1.3	0.44
		Glomeromycota	0.7 ± 0.2	0.9 ± 0.2	>0.5
		Zygomycota	2 ± 2	1.6 ± 0.6	>0.5
		Unidentified	52.6 ± 4.3	40.3 ± 13	0.1

[1] Relative abundances were calculated of the rarefied dataset. BS= Bulk soil, R= Rhizosphere.

[2] Assessed using the Mann-Whitney U-test (two-tailed, P ≤ 0.05). Significant comparisons are highlighted in bold.

In summary, in both experiments endophyte infection had only a minor impact on prokaryotic community composition in the rhizosphere of their hosts. The only significant effects identified at the level of phyla were a 1.1% increase in the relative abundance of Bacteroidetes in infected versus uninfected plants, although only in the first experiment. At the genus level, in both experiments rhizosphere communities of infected and uninfected plants appeared very similar. At the level of OTUs there was one significantly differentially abundant OTU between rhizospheres of infected and uninfected plants in the first experiment and nine in the second- although all were present in relatively low relative abundances (<0.3%), and none were significantly differentially abundant in both experiments. For fungi, effects of endophyte infection consistent in both experiments was limited to a single abundant highly enriched in the rhizosphere compared to bulk soils which was present in decreased abundances in the rhizosphere of infected versus uninfected plants in both experiments. While the significance of this difference could not be shown in individual experiments, fitting the data from both experiments to a linear regression model accounting for experimental variation found that the endophyte-associated decrease was significant across both experiments. QIIME assigned this OTU as belonging to the genus *Ceratocystis,* although this was based only on an alignment of 90 nucleotides to three *Ceratocystis* species at the beginning of the total 266 nucleotides of the representative sequence of the OTU. However, >95% of the total OTU sequence mapped with 76-90% similarity to a number of deposited sequences assigned as belonging to the class Sordariomycetes, or species within this class (Appendix A8). Comparison of the degree of differentiation of rhizosphere communities between each experiment irrespective of endophyte infection versus between endophyte infected versus uninfected plants in each experiment found that the greater extent of differentiation in prokaryotic and fungal community composition of plants of each experiment observed in beta-diversity analyses were largely due to differences at the OTU level. As expected, there were also a number of consistently differentially abundant prokaryotic and fungal phyla between bulk soil and rhizosphere communities in both experiments, although in some cases the significance of these differences were demonstrable only in the first experiment (Table 6).

3.5.5 Targeted analyses of arbuscular mycorrhiza

As outlined in the introduction, several previous studies have shown positive (Arrieta et al., 2015; Vignale et al., 2015) as well as negative (Chu-Chou et al., 1992; Liu et al., 2011; Muller, 2003) effects of *Epichloë* endophyte infection towards certain arbuscular mycorrhizal (AM) fungi. Given the very low proportion of Glomeromycota reads present in the fungal dataset, it was hypothesized that there could still be effects towards AM that would not be obvious in the analysis conducted on the total fungal dataset. To assess for effects towards AM in this study, reads assigned to Glomeromycota were filtered from the original unrarefied ITS OTU table, and this new OTU table was rarefied such that each sample contained the same number of reads as the sample with the lowest number of reads (73 reads per sample) (Figure 21). In all cases, methods used in these analyses were identical to those carried out on complete prokaryotic and fungal datasets.

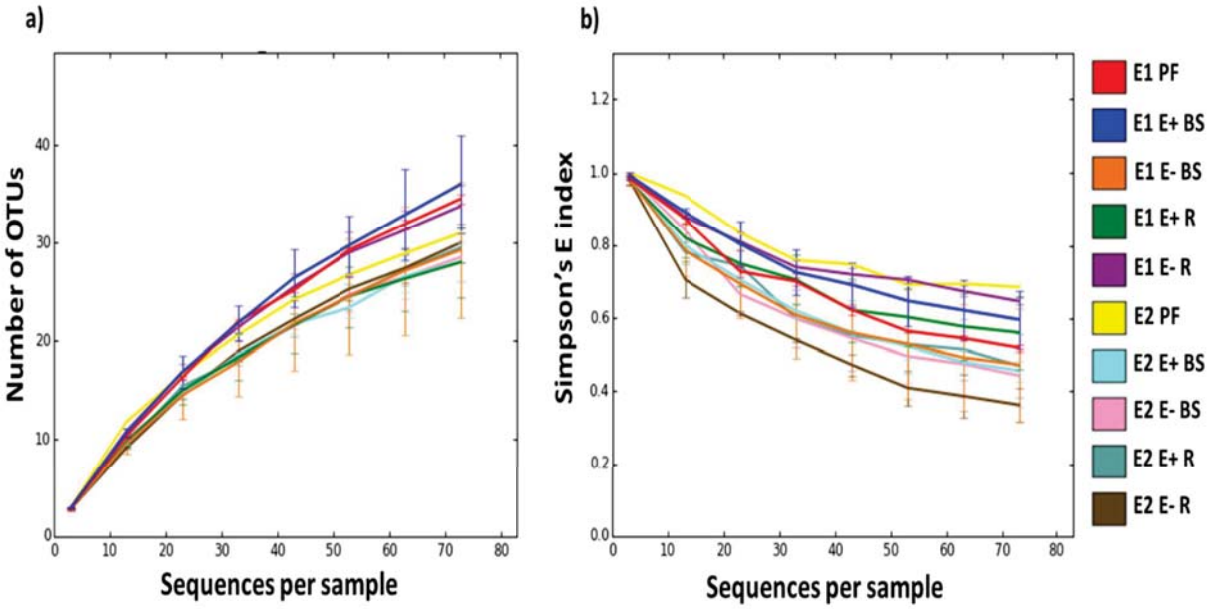

Figure 21. Rarefaction curves of AM. a) Species richness (number of OTUs) and **b)** Species evenness (Simpson's E index) clustered by experiment, sample type and endophyte status. Error bars indicate standard deviations of each treatment. PF= Plant-free; BS= Bulk soil; R= Rhizosphere. E1/E2= experiment one/two, E+/-= endophyte-infected/uninfected.

3.5.5.1 No significant effects of endophyte infection on alpha-diversity of the AM community in the rhizosphere

Similarly to that of prokaryotes and all fungi, endophyte infection had no significant effect on species richness or evenness of AM in the rhizosphere in either experiment (Figure 22 e, f). Additionally, there were no significant differences in alpha-diversity of AM rhizosphere communities between each experiment, nor between bulk soil and rhizosphere communities within each experiment (Figure 22 b, c).

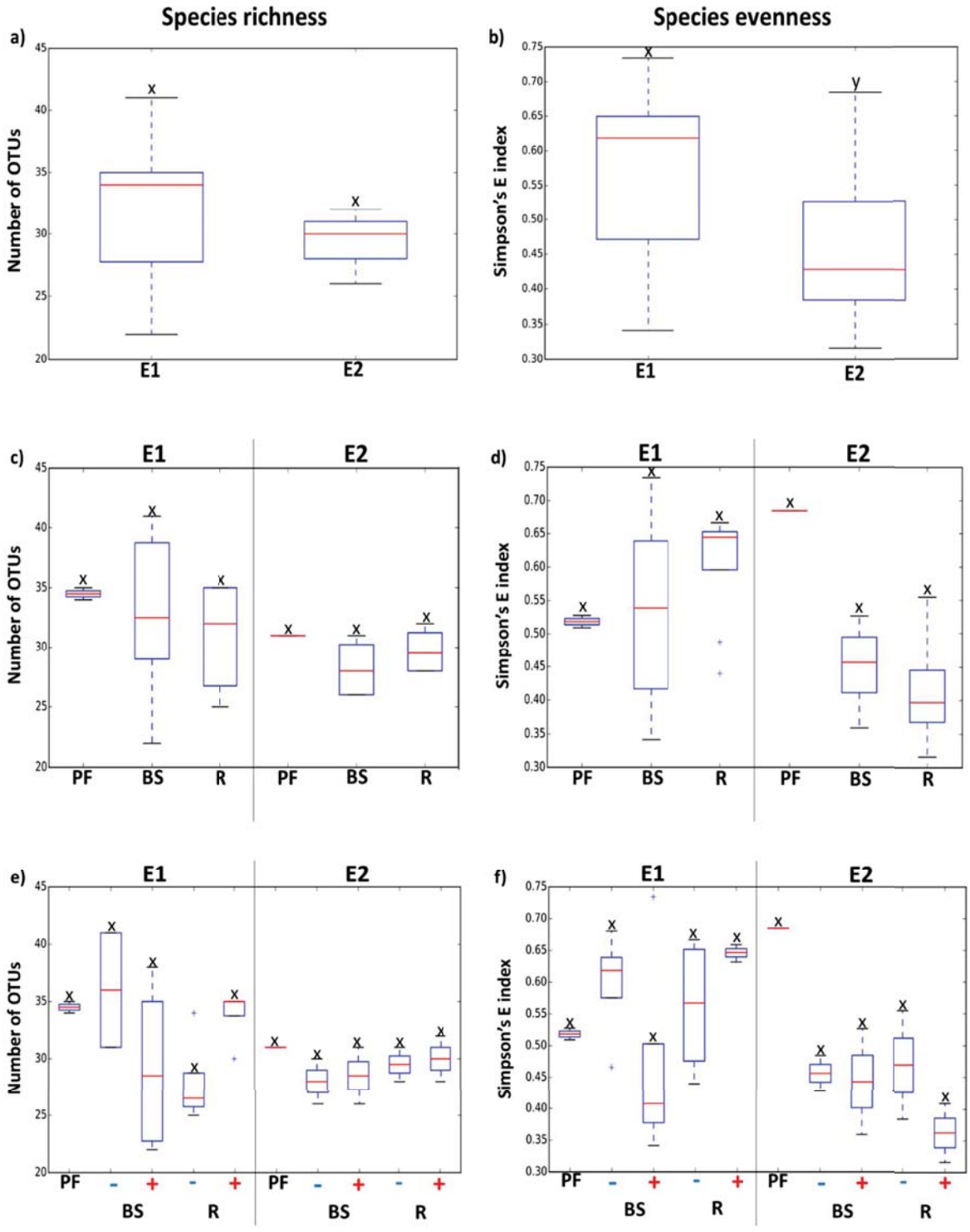

Figure 22. Alpha-diversity of AM communities. Box-plots of species richness (a, c, e) and evenness (b, d, f) of AM samples clustered by experiment (a, b), experiment and sample type (c, d), and experiment, sample type and endophyte status (e, f). Categories displaying a different letter above them were statistically significant (P<0.05; non-parametric two-sample T-test) from one another.PF=plant-free; BS= Bulk soil; R=Rhizosphere; red crosses/blue dashes=endophyte infected/uninfected.

3.5.5.2 No significant impact of endophyte infection on overall community composition of arbuscular mycorrhiza in the rhizosphere

To assess whether endophyte infection impacted overall community composition (beta-diversity) of AM communities in the rhizosphere of their hosts, principal coordinate analysis (PCoA) plots of Bray-Curtis dissimilarity distances of AM communities were generated (Figure 23 a). In both experiments, no significant differences were identified between rhizosphere communities of infected and uninfected plants. However, samples of experiment one and experiment two formed significantly distinct clusters (Figure 23 b), both irrespective of sample type as well as between bulk soil and rhizosphere communities between experiments (Table 7). This shows that even at a sequence depth as low as 73 reads per sample, a highly significant difference was still detectable between AM communities present in each experiment. Bulk soil and rhizosphere samples also formed significantly distinct clusters in the first experiment, but not the second (Table 7).

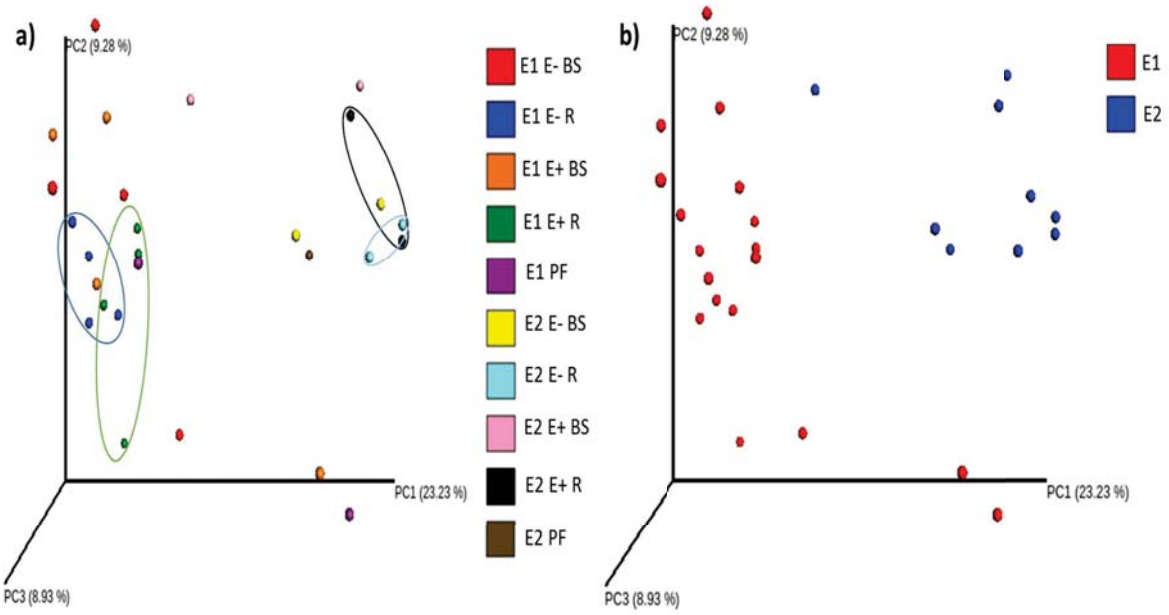

Figure 23. Beta-diversity of AM communities. PCoA plots of Bray-Curtis dissimilarity distances between AM communities of each sample, with samples grouped by **a)** experiment, sample type and endophyte status and **b)** only experiment. Percentages on each axis represents the percentage of variation displayed along that particular axis.

Table 7. PERMANOVA comparisons of beta-diversity of AM fungi.

Comparison[1]	Pseudo-F	P-value[2]
E1 vs E2	**5.36**	**0.001**
E1 BS vs E2 BS	**2.6**	**0.003**
E1 R vs E2 R	**5.66**	**0.002**
E1 BS vs E1 R	**2.32**	**0.002**
E1 E+ BS vs E1 E- BS	1.08	0.44
E1 E+ R vs E1 E- R	1.02	0.35
E2 BS vs E2 R	1.36	0.17
E2 E+ BS vs E2 E- BS	0.96	0.68
E2 E+ R vs E2 E- R	0.5	1.0

[1] E1/E2= Experiment one/two, BS= Bulk soil, R= Rhizosphere, E+/-= Endophyte-infected/uninfected.

[2] False discovery rate (FDR)-corrected. Statistically significant comparisons ($P \leq 0.05$) are highlighted in bold.

3.5.5.3 Endophyte-associated promotion of Acaulosporaceae in the first experiment but not the second

To gain further insight into the degree of differentiation in taxonomic composition of AM communities in each sample, relative abundance taxa plots were generated (Figure 24). Given the findings of the above beta-diversity analysis, it was hypothesized that AM rhizosphere communities would differ more so in their overall composition between samples of each experiment than they would between infected and uninfected plants within each experiment.

In the first experiment, Acaulosporaceae was present in significantly higher relative abundances in the rhizosphere of infected plants compared with uninfected plants (21.9 ± 7.5% in E+ versus 7.5 ± 2.6% in E-; *P*= 0.029 (Mann-Whitney U test)), however this was not observed in the second experiment. (Table 9). Aside from this there was also an observable decrease in Archaeosporaceae and unidentified Glomeromycota in the rhizosphere of infected compared with uninfected plants (Figure 24 a), although these differences were not statistically significant (Table 8).

To better visualise the extent of variability in community composition of AM communities between each experiment compared with between endophyte treatments within each experiment, taxa plots of rhizosphere samples collapsed into single plots based on their endophyte status within each experiment, as well as between each experiment were generated (Figure 24 b). The only significantly differentially abundant phyla between rhizosphere samples of each experiment was a 25.6% increase in the relative abundance of unidentified Paraglomerales in E2 compared with E1. While their significance could not be shown, there were also apparent differences in the relative abundances of Acaulosporaceae and Gigasporaceae in the rhizosphere of plants between each experiment (Table 9). As expected, this shows that overall differences in taxonomic composition of AM communities was greater between the rhizospheres of plants of each experiment irrespective of endophyte infection than between plants of differing endophyte status within each experiment.

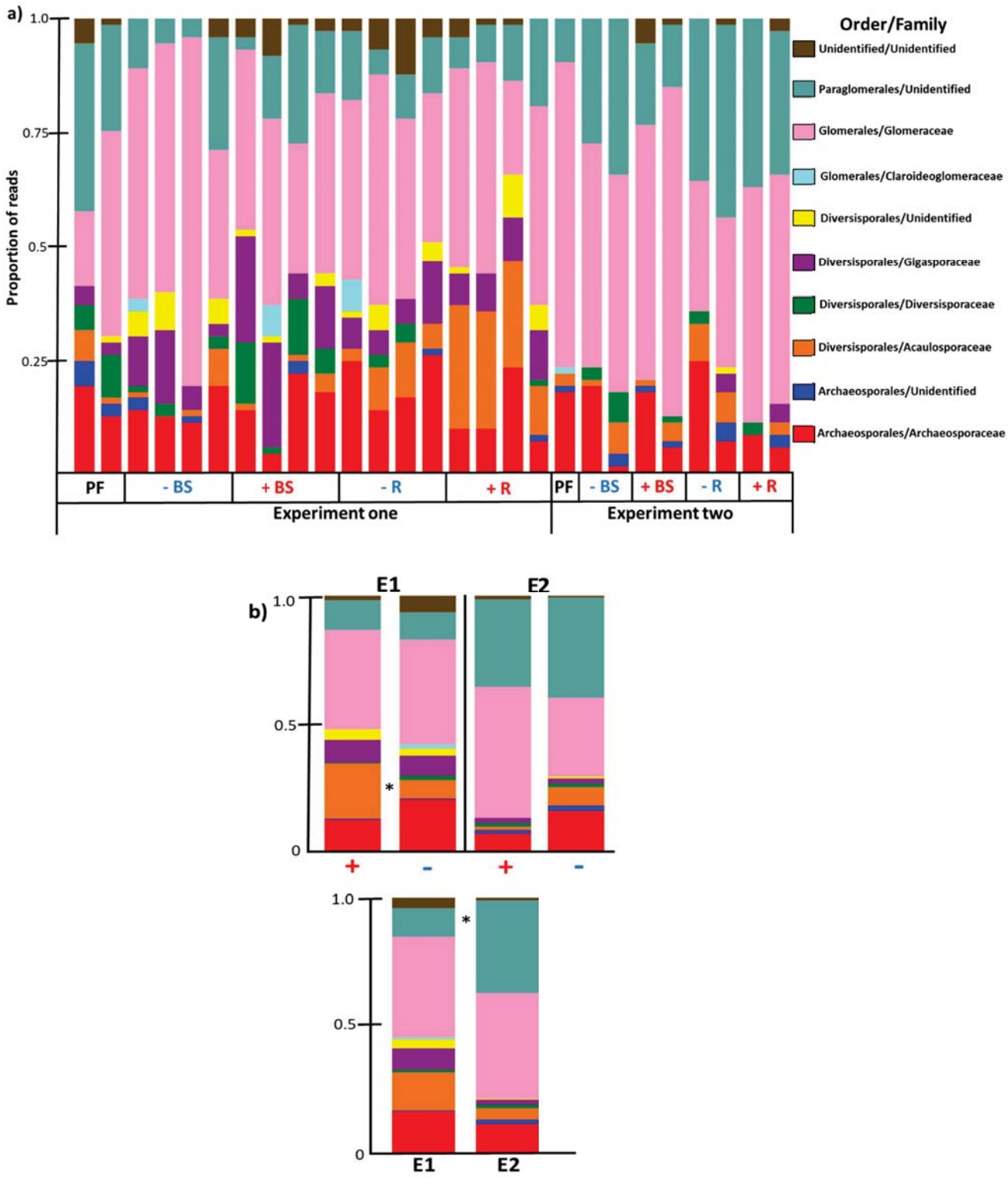

Figure 24. Relative abundance taxa plots of AM families. a) Taxa plots of each individual sample. **b)** Taxa plots of samples collapsed into single plots based on endophyte status within experiments, and all rhizosphere samples of each experiment. E1/E2= Experiment one/two; PF= Plant-free; BS= Bulk soil; R= Rhizosphere; red/blue font= endophyte-infected/uninfected.

Table 8. Relative abundances of Glomeromycotan families in rhizosphere samples of infected versus uninfected plants.

Experiment	Order/Family	E+ R relative abundance (%)	E- R relative abundance (%)	P-value[2]
1	Archaeosporales/Archaeosporaceae	12.3 ± 7.4	20.2 ± 6.1	0.3
	Archaeosporales/Unidentified	0.4 ± 0.7	0.4 ± 0.7	>0.5
	Diversisporales/Acaulosporaceae	**21.9 ± 7.5**	**7.5 ± 4.3**	**0.029**
	Diversisporales/Diversisporaceae	0.4 ± 0.7	1.7 ± 2	>0.5
	Diversisporales/Gigasporaceae	8.9 ± 1.8	7.9 ± 3.9	>0.5
	Diversisporales/Unidentified	4.1 ± 4.3	2.8 ± 2.5	>0.5
	Glomerales/Glomeraceae	38.7 ± 12.2	40.8 ± 7.4	>0.5
	Paraglomerales/Unidentified	11.6 ± 5.6	10.6 ± 4.1	>0.5
	Unidentified	1.7 ± 1.7	6.5 ± 4.2	>0.5
2	Archaeosporales/Archaeosporaceae	6.9 ± 1.9	15.8 ± 12.7	n/a
	Archaeosporales/Unidentified	1.4 ± 1.9	2.1 ± 2.9	n/a
	Diversisporales/Acaulosporaceae	1.4 ± 1.9	7.5 ± 1	n/a
	Diversisporales/Diversisporaceae	1.4 ± 1.9	1.4 ± 1.9	n/a
	Diversisporales/Gigasporaceae	2.1 ± 2.9	2.1 ± 2.9	n/a
	Diversisporales/Unidentified	0	0.7 ± 1	n/a
	Glomerales/Glomeraceae	51.4 ± 1	30.9 ± 2.9	n/a
	Paraglomerales/Unidentified	34.3 ± 3.9	39.1 ± 4.9	n/a
	Unidentified	1.4 ± 1.9	0.7 ± 1	n/a

[1] E+/E- = Endophyte-infected/uninfected; R= Rhizosphere.

[2] Significance was assessed using the Mann-Whitney U-test (two-tailed, $P \leq 0.05$).

Significant comparisons are highlighted in bold.

Table 9. Relative abundances of Glomeromycotan families between each experiment.

Comparison[1]	Order/Family	E1 relative abundance (%)	E2 relative abundance (%)	P-value[2]
E1R vs E2R	Archaeosporales/Archaeosporaceae	16.3 ± 7.6	11.3 ± 9	>0.5
	Archaeosporales/Unidentified	0.4 ± 0.6	1.7 ± 2	>0.5
	Diversisporales/Acaulosporaceae	14.7 ± 9.5	4.4 ± 3.8	0.29
	Diversisporales/Diversisporaceae	1 ± 1.6	1.4 ± 1.6	>0.5
	Diversisporales/Gigasporaceae	8.4 ± 2.9	2.1 ± 2.4	>0.5
	Diversisporales/Unidentified	3.4 ± 3.4	0.4 ± 0.7	>0.5
	Glomerales/Glomeraceae	39.7 ± 9.4	41.1 ± 12	>0.5
	Paraglomerales/Unidentified	**11.1 ± 4.6**	**36.7 ± 4.5**	**0.024**
	Unidentified	4.1 ± 3.9	1 ± 1.3	>0.5
E1BS vs E2BS	Archaeosporales/Archaeosporaceae	14.2 ± 5.5	11 ± 8.9	>0.5
	Archaeosporales/Unidentified	0.9 ± 1.2	1.4 ± 1.1	>0.5
	Diversisporales/Acaulosporaceae	2.2 ± 2.7	3.4 ± 2.6	>0.5
	Diversisporales/Diversisporaceae	5 ± 5.2	2.7 ± 2.9	>0.5
	Diversisporales/Gigasporaceae	12.6 ± 8	0	n/a
	Diversisporales/Unidentified	3.1 ± 3	0	n/a
	Glomerales/Glomeraceae	45.6 ± 15.2	56.5 ± 11.3	0.29
	Paraglomerales/Unidentified	12.7 ± 8.9	23.3 ± 9.3	0.34
	Unidentified	2.6 ± 2.9	1.7 ± 2.6	>0.5
All E1 vs All E2	Archaeosporales/Archaeosporaceae	15.3 ± 6.2	11.9 ± 8.1	0.25
	Archaeosporales/Unidentified	1 ± 1.5	1.5 ± 1.4	0.3
	Diversisporales/Acaulosporaceae	8 ± 8.9	3.8 ± 2.9	0.45
	Diversisporales/Diversisporaceae	3.5 ± 4.3	1.8 ± 2.3	>0.5
	Diversisporales/Gigasporaceae	**9.7 ± 6.3**	**0.9 ± 1.8**	**0.001**
	Diversisporales/Unidentified	**3 ± 3**	**0.2 ± 0.5**	**0.006**
	Glomerales/Glomeraceae	41.3 ± 13.3	50.8 ± 14.1	0.07
	Paraglomerales/Unidentified	**13.9 ± 9**	**27.7 ± 11.4**	**0.007**
	Unidentified	3.3 ± 3.3	1.2 ± 1.9	0.07

[1] E1R vs E2R= all rhizosphere samples of experiment one versus all rhizosphere samples from experiment two; E1BS vs E2BS= all bulk soil samples of experiment one versus all rhizosphere samples from experiment two (excluding samples from plant-free controls); All E1 vs All E2= All samples of experiment one versus all samples from experiment two, including samples from plant-free controls.

[2] Significance was assessed using the Mann-Whitney U-test (two-tailed, P≤0.05). Significant comparisons are highlighted in bold.

Overall, targeted analyses of fungal reads that mapped to AM found that endophyte infection did not significantly affect overall alpha or beta-diversity of the rhizosphere

community, however rhizospheres of infected plants had a significantly higher relative abundance of species belonging to the family Acaulosporaceae than uninfected plants in the first experiment but not the second. In the second experiment, the rhizosphere microbiomes of the two endophyte-infected plants contained lower levels of Acaulosporaceae than those of the two uninfected plants. However, it is important to note that this analysis was carried out at the low sequence depth of 73 reads per sample due to the very small proportion (<0.01%) of total ITS reads that mapped to Glomeromycota in this study. Nevertheless, even at this low sequence depth, these results clearly illustrate that the effects of experimental variation on AM community composition appear larger than that of endophyte infection.

4 | Discussion and Conclusion

Despite being virtually completely absent from root tissue (Christensen and Voisey 2007), infection of cool-season grasses with *Epichloë* endophytes has been shown to impact biogeochemical processes as well as microbial community composition in the rhizosphere of their hosts (see section 1.2.3). Although this had not been previously assessed in the symbiosis between perennial ryegrass (NuiD) and its natural endophyte *E. festucae* var. *lolii* (Lp19), the recent observation of endophyte-associated impacts on the root transcriptome (Schmid et al, 2017) led to the hypothesis that the rhizosphere microbiome of perennial ryegrass could undergo significant alterations as a consequence of endophyte infection in this particular interaction. Surprisingly however, under the experimental conditions used in this study, consistent effects of endophyte infection on rhizosphere community composition were very limited. A small number of endophyte-associated effects were observed in each experiment, although these generally only occurred in one of the two experiments of this study. The one exception was a reduction in frequency of reads mapping to an abundant OTU likely of the class Sordariomycetes that was highly enriched in the rhizosphere. In contrast, greater differences in both prokaryotic and fungal alpha diversity and community composition in the rhizosphere were shown between rhizospheres of plants that were grown on two separate occasions, thereby further emphasizing the variability of endophyte-associated effects towards the prokaryotic and fungal rhizosphere microbiome in comparison to those brought about by other growth parameters.

4.1 Minor impact of endophyte infection on prokaryotic rhizosphere community composition

In both experiments of this study, the prokaryotic rhizosphere microbiome of perennial ryegrass was only very mildly impacted by *E. festucae* var. *lolii* infection. Alpha-diversity analyses showed that endophyte infection did not significantly affect species richness or evenness of the rhizosphere microbiomes of their hosts in either experiment. Beta-diversity analyses illustrated a very mild but significant shift in the overall composition of the rhizosphere community, although only in the first experiment. At the level of phyla, while there were some minor impacts of endophyte infection between rhizospheres of

infected and uninfected plants, the only statistically significant difference was a minor 1.1% increase in Bacteroidetes in the rhizosphere of infected versus uninfected plants. At the genus level, in both experiments taxonomic profiles of rhizospheres of infected and uninfected plants appeared very similar, and displayed little variability between replicates. At the level of individual OTUs, only one prokaryotic OTU was differentially abundant depending on endophyte status in the first experiment and nine in the second, all of which were present in very low relative abundances (<0.3%). There were also no prokaryotic OTUs that were consistently significantly differentially abundant in both experiments. Nevertheless, despite being minuscule in nature, due to the importance of the rare biosphere (Jousset et al., 2017) (see section 1.1.2.2) it is entirely possible that these differentially abundant OTUs could still potentially have important implications on the development and physiology of their hosts. Despite our finding of these effects not having any obvious morphological effects towards their hosts, it is also possible that these could have induced effects towards their hosts that were not detectable at the phenotypic level.

Previous analyses of the prokaryotic rhizosphere microbiome of cool-season grasses in response to infection with *Epichloë* endophytes have generally also displayed only subtle but significant endophyte-associated effects rather than widespread alterations to the rhizosphere community. Wakelin et al (2015) found shifts in the overall bacterial rhizosphere community of perennial ryegrass plants infected with the commercialized *E. festucae var. lolii* strains AR1 and AR37, however specific analysis of *Pseudomonas* species found no effect towards this genus. Roberts and Ferraro (2015) also found that tall-fescue plants infected with *E. coenophiala* contained higher levels of species richness and 8.8% higher relative abundances of Firmicutes in their rhizospheres compared with uninfected plants (14.6% in infected versus 5.8% in uninfected). On the other hand, another recent metabarcoding analysis of the rhizosphere microbiome of tall-fescue in response to *E. coenophiala* infection found no significant effects of endophyte infection towards the prokaryotic rhizosphere microbiome of their hosts (Rojas et al., 2016).

In addition to the abovementioned studies which enriched for rhizosphere soil, it has also been shown that endophyte-associated effects towards belowground soil communities without enriching for rhizosphere soil. Bell et al (2009) sampled soil cores (four 5 mm

diameter x 70 mm deep mini-cores from each pot) from pots containing plants infected with three endophyte strains: Lp19 (this study), AR1, and AR37, and assessed for differences in community composition of particular bacterial groups using Denaturing Gradient Gel Electrophoresis (DGGE). This study found that infection with Lp19 was associated with significant shifts in *Pseudomonas* and *Actinobacteria* bulk soil communities. Another study by Casas et al (2011) also sampled the bulk soil (6 cm diameter, 2-8 cm deep from each pot) in association with Italian ryegrass (*Lolium multiflorum*) plants infected or uninfected with *Epichloë occultans*, and found a subtle but significant shift in bacterial communities between soil cores collected underneath infected and uninfected plants. Given that no significant differences in bulk soil communities of infected and uninfected replicates were observed, our results contrast those of these studies.

Despite our results suggesting a very minor impact of endophyte infection towards prokaryotic community composition of their hosts, effects of endophyte infection could be more pronounced in other environmental contexts. As also posited by Rojas et al (2016), endophyte-associated effects towards the rhizosphere of their hosts could be greater under stressful conditions. Similarly, it is also possible that effects of *Epichloë*-associated effects may take longer periods of time to develop than the ~two months of growth of these experiments, and could also become more pronounced in densely populated pastoral settings compared to the individually growing replicates analysed in this study.

4.2 Endophyte-associated effects towards rhizosphere fungi largely limited to a single abundant OTU

Endophyte-associated impacts towards most rhizosphere fungi were minor, and the vast majority of apparent effects were not observed in both experiments. The only exception to this was an endophyte-associated reduction observed toward an abundant fungal OTU that was highly enriched in rhizosphere samples relative to bulk soil. While the significance in the differential abundance of this OTU could not be demonstrated in each experiment, fitting the data from both experiments to a regression model to control for experimental

variability found that this endophyte-associated reduction was statistically significant across both experiments.

Given the inability of ITS marker sequences to accurately predict taxonomy to the level of species (Blaalid et al., 2013), it is difficult to ascertain the biological impact that this endophyte-associated antagonism could potentially have on their hosts. Sordariomycetes are a fungal class comprising a diverse range of plant beneficial as well as fungal pathogens- some of which are known to infect ryegrass roots (Harmon & Latin, 2005; Skipp & Christensen, 1989). The lack of any significant differences identified in aboveground biomass or tillering rates of plants in both experiments of this study (section 3.2.3) suggests that the species represented by this OTU are likely commensal towards ryegrass. However, rather than directly affecting perennial ryegrass plants, it is also possible that the species represented by this OTU could potentially use the rhizosphere of ryegrass as a reservoir to maintain their abundances in the soil biome. For example, the rhizosphere microbiome of some crops have been shown to act as a reservoir for human pathogens such as *Salmonella* and enterohemorrhagic *E. coli*, which thereafter recolonize human hosts when their plant hosts are introduced into the food chain (Mendes et al., 2013). In a similar vein, the species represented by this OTU may use the rhizosphere of perennial ryegrass as a means of maintaining their abundances in the soil biome until a susceptible host is in close proximity. Nevertheless, without further work involving more targeted means of assessing which particular species are represented by this OTU, the biological impact of its reduction can only be speculated.

The endophyte-associated reduction in abundances of this OTU could occur via a range of different mechanisms, either as the direct result of compounds secreted by the endophyte or alternatively through endophyte-induced changes in the gene expression (Dupont et al., 2015; Schmid et al., 2017) or metabolism (Rasmussen et al., 2008; Wakelin et al., 2015) of their host plants. For example, in *E. coenophiala*-infected tall-fescue, lolines produced by the endophyte are translocated to the roots which exerted selection pressure towards two loline-catabolizing microorganisms (Roberts and Ferraro, 2015). While the endophyte assessed in this study does not produce lolines (Johnson et al., 2013), peramine has been detected in roots of infected plants- albeit at much lower concentrations than in foliar plant parts (Fannin et al., 1990). Given the recent findings of both endophyte-associated impacts

on the root transcriptome (Schmid et al., 2017) as well as the rhizosphere metabolome (Wakelin et al., 2015) in perennial ryegrass, such impacts could make the rhizosphere microbiome less accommodating to the species represented by this OTU.

To date, there have been fewer cultivation-independent analyses that have assessed the effects of *Epichloë* endophytes towards fungal rhizosphere community composition than there have been for prokaryotes. Despite the endophyte-associated effects towards towards the OTU belonging to the class Sordariomycetes observed in this study, the results of beta-diversity analyses suggesting that overall community composition of the rhizosphere microbiome of their hosts was not significantly affected by endophyte infection contrasts the results of previous analyses that have assessed identified endophyte-associated shifts in fungal rhizosphere communities. Wakelin et al (2015) found *E. festucae* var. *lolii* infection of perennial ryegrass had a subtle but significant effect towards the fungal rhizosphere community of plants infected with the commercial strains AR1 and AR37. Rojas et al (2016) found subtle but marginally significant decreases in Ascomycota and increases in Glomeromycota in *E. coenophiala*-infected tall fescue plants compared with uninfected plants. While non-significant, endophyte-associated decreases in Ascomycota were also observed in both experiments of this study. Our finding of no significant effects of endophyte infection aligns with Casas et al (2011) which found no significant impacts towards fungal communities in *Epichloë occultans*-infected Italian ryegrass (*Lolium multiflorum*)- although Casas et al (2011) did not specifically enrich for rhizosphere soil as was carried out in this study, and instead only sampled soil cores underneath growing plants.

Given the previous effects *Epichloë* endophyte infection has been shown to have towards some arbuscular mycorrhizal (AM) fungi (see section 1.2.3.1), it was hypothesized that targeted analyses of AM reads from the fungal dataset may illustrate endophyte-associated effects. However, instead there were no significant effects of endophyte infection towards alpha or beta diversity of AM communities in the rhizosphere of their hosts in either experiment. At the family level, the only significant endophyte-associated effect observed was a 14.4% increase in the relative abundance of Acaulosporaceae that occurred in the first experiment but not the second.

Previous analyses showing *Epichloë*-associated effects towards AM have typically involved more targeted analyses than that of this study, using techniques that allow for accurate quantification of particular AM species. For example, early studies examining these effects in *E. coenophiala*-infected plants involved microscopy-based methods such as the counting of spores on plant roots (Chu-Chou et al., 1993; Guo et al., 1992). More recently, quantitative PCR (qPCR)-based methods have been used to illustrate effects of endophyte infection towards two particular *Glomus* species (Liu et al., 2011). Our results therefore do not necessarily refute those of these studies, as the more holistic assessment carried out in this study was unable of providing quantitatively accurate measures of the biomass of single AM species and therefore do not prove the absence of these effects. This is especially so given that the analysis in this study was carried out at the relatively low sequence depth of 73 reads per sample, due to the low proportion of sequences assigned as belonging to AM generated by the PCR primers used. Given that high phosphorus levels are known to inhibit AM (Breuillin et al., 2010; Menge et al., 1978), the high levels of phosphorus in the soil used in this study may have also contributed to the lack of any consistently observed effects.

4.3 Endophyte-associated impacts on the rhizosphere microbiome were dwarfed by effects due to variation between experiments

Aside from the notable exception of the differential abundance of the abovementioned abundant fungal OTU, analyses consistently found that both prokaryotic and fungal rhizosphere communities differed greater between plants of each independent experiment than between rhizospheres of infected and uninfected plants within each experiment. Alpha-diversity analyses showed that both prokaryotic and fungal species richness were significantly greater in the first experiment than in the second, and prokaryotic species evenness in the rhizosphere was also greater in experiment two than experiment one for prokaryotes but not fungi. Beta-diversity analyses showed highly significant clustering of prokaryotic and fungal rhizosphere samples depending on which experiment they were from. Taxonomic analyses found that while there were few significant differences in the relative abundances of phyla between experiments, at the

level of individual OTUs, a far larger number of prokaryotic and fungal OTUs were significantly differentially abundant between rhizospheres of each experiment than were found between plants of differing endophyte status within each experiment. Finally, targeted analyses of Glomeromycota found highly significant clustering of AM rhizosphere communities between each experiment. Overall, while differences in rhizosphere community composition of plants of each experiment were not particularly drastic, the difference was nevertheless greater than that due to endophyte infection within both experiments.

The two experiments of this study examined the same genotypic symbiotic host-endophyte interaction in soil collected from the same site, although at different times of the year. Watering regimes and soil compaction levels also differed between experiments, as well as the exposure of plants to insects and insecticides (see section 3.2). Due to multiple variables differing between each experiment, it cannot be precisely pinpointed which of these accounted for the observed differences in rhizosphere community composition between each experiment. For example, these differences could have been driven by seasonal variation (Dumbrell et al., 2011; Shi et al., 2015; Voriskova et al., 2014) of the soil collected from the site between each time of collection, and/or due to differences in other variables such as exposure to insecticides (Jacobsen & Hjelmsø, 2014) and soil compaction levels (Hartmann et al., 2014) that plants of each experiment were subjected to. Differences in microbial community composition during rainy versus dry seasons have been previously shown (Nessner Kavamura et al., 2013; Torres-Cortés et al., 2012) making it possible that the slightly different watering regimes that were used in each experiment could have also contributed to these differences. It is also possible that animal grazing that may have occurred between collection of soil for each experiment could have had impacted the resident soil biome of the site (Vargas et al., 2015).

The finding of comparatively greater differences in rhizosphere community composition between each experiment of this study suggests that the impacts of endophyte infection observed in this study would likely be dwarfed by effects caused by other biotic and abiotic variability that plants would typically face while growing in uncontrolled field conditions. However, this only holds true under the assumption that effects of endophyte infection would be as subtle under field conditions as was observed in the plants grown under the

controlled conditions of this study. As already mentioned, it is plausible to speculate that endophyte-associated impacts on the rhizosphere microbiome of their hosts could be greater in different environmental contexts- particularly in plants growing under stressful conditions. This hypothesis is supported by our finding of an apparently greater effect of endophyte infection towards rhizosphere fungi in the second experiment, where plants did not grow well and appeared physiologically more stressed than the plants in the first experiment. However, given only two biological replicates were analysed in the second experiment the statistical significance of this effect was not demonstrable. Regardless, these results suggest that other biotic and abiotic parameters have a greater impact on rhizosphere community composition than endophyte infection (with the exception of the abovementioned abundant fungal OTU, which was instead more greatly affected by endophyte infection).

4.4 Structural differentiation of the rhizosphere microbiome from bulk soil irrespective of endophyte infection

Surprisingly, prokaryotic rhizosphere communities had significantly higher species richness than in bulk soil in both experiments. Given that microbial communities in the root microbiome typically decrease in diversity with increasing proximity to the root (Bulgarelli et al., 2013; Vandenkoornhuyse et al., 2015), it is unclear why this opposite trend was observed in this study. While it can only be speculated, perhaps the rhizosphere sampling procedure used in this study may have contributed to this observed peculiarity. Rather than being consistent in community composition, roots are known to harbour different microsites of microbial communities throughout a given plant root system (Marschner et al., 2002; Semenov et al., 1999). Given this, different sections of the root may have selected for and allowed the proliferation of different microbes otherwise present in the bulk soil at levels too low to be detected at the sequence depths which datasets were rarefied to in this study. Due to the rhizosphere sampling procedure washing entire root systems in buffer followed by centrifugation of this buffer to pellet rhizosphere soil, this procedure would have condensed DNA from throughout the entirety of the root system, potentially underlying the increased species richness in rhizosphere samples compared with the bulk

soil. This possibility is supported by the fact that the study that the rhizosphere sampling procedure of this study was based on also found slightly higher alpha diversity of communities in the rhizosphere than bulk soil in some soils, and similar levels of diversity in others (Edwards et al., 2015). In contrast, the sampling procedure used for bulk soil of simply taking a few subsamples throughout the tube may not have adequately represented the entirety of diversity present in the bulk soil. To mitigate this in future experiments, a better strategy of sampling bulk soil may be to mix all bulk soil within each replicate thoroughly prior to pooling multiple subsamples for DNA extractions, so that the extracted DNA better represents the overall diversity of the bulk soil used rather than merely a few microsites therein.

While this is the first high-throughput sequencing study to compare the rhizospheres of perennial ryegrass plants infected or uninfected with *Epichloë* endophytes, there have been two previous studies that have assessed the bacterial communities in the rhizosphere of uninfected perennial ryegrass plants. Lagos et al (2014) found that rhizospheres of plants grown under controlled conditions for one month in two different Chilean Andisol soils were dominated by Proteobacteria, Actinobacteria and Acidobacteria, as was also seen in this study. However, rhizospheres of plants contained lower relative abundances of Verrucomicrobia and Planctomycetes compared to this study. Notably, the soils used contained far lower levels of phosphorus than that used in this study (19 & 2 mg/L versus 97 & 109 mg/L in this study). More recently, Chen et al (2016) analysed the root microbiomes of perennial ryegrass plants growing in two different soils under two different carbon dioxide levels under controlled conditions and found that rhizospheres were largely dominated by Proteobacteria, with relative abundances approximately twice as high as was seen in this study. While not necessarily surprising, the degree of differentiation in rhizosphere community composition between each of these studies illustrates the dynamic nature of the microbiome of perennial ryegrass when different cultivars are growing in different soils. However, variability in methods used to sample the rhizosphere (Berg & Smalla, 2009), DNA extraction protocols (Brooks et al., 2015), PCR primers (Peiffer et al., 2013), and bioinformatics softwares (Clooney et al., 2016) that were used to process the sequence data generated in each study would have also invariably contributed to these differences in results between these studies.

To our knowledge, this is the first metabarcoding analysis of the fungal rhizosphere microbiome of perennial ryegrass. However, the results of this study align with a recent metabarcoding study that assessed the fungal rhizosphere microbiome of tall-fescue, in that Chytridiomycota were present in significantly higher relative abundances in the rhizosphere than in bulk soil. In comparison with Rojas et al (2016) where only Chytridiomycota and Zygomycota significantly differed in their abundances between the rhizosphere and bulk soil, in this study all fungal phyla aside from Zygomycota (Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota) were significantly differentially abundant between bulk soil and rhizosphere samples in experiment one. While non-significant, aside from Glomeromycota, these same trends were all also observed in the second experiment. One potential explanation underlying the comparatively stronger differentiation between bulk soil and rhizosphere communities observed in this study could have been due to the different methods implemented in each study to sample the rhizosphere. Rojas et al (2016) did so by brushing soil from roots with a sterile paintbrush, whereas in this study loose soil was completely removed from roots and thereafter washed in buffer to enrich for soil very close to the root surface. As a result, the procedure used to sample the rhizosphere in this study may have more strongly enriched for the rhizosphere than that used in Rojas et al (2016), thereby potentially underlying why differences in community composition between rhizosphere and bulk soil communities were more pronounced in this study.

One particularly drastic difference observed between bulk soil and rhizosphere communities was in the relative abundances of the OTU likely representing species of the class Sordariomycetes that was found to be present in significantly lower abundances in the rhizosphere of infected versus uninfected plants. In both experiments, this OTU was present in an average 26-fold greater relative abundance in rhizosphere samples than bulk soil in plants across both experiments. This suggests that through some mechanism, the species represented by this OTU is capable of flourishing in the rhizosphere of perennial ryegrass despite only being present in low relative abundances in the wider bulk soil. The mechanism responsible could possibly involve recruitment from the plant, similarly to that seen in the recruitment of beneficial rhizosphere bacteria through the secretion of malic acid by *Arabidopsis thaliana* roots (Rudrappa et al., 2008). Alternatively, the species

represented by this OTU may possess specialized means of invading the rhizosphere environment and out-competing other fungi without being actively recruited by the plant. Until further work involving more targeted analyses are carried out the underlying mechanism of this enrichment can only be speculated. However, this observation highlights that the high abundance of this OTU in the rhizosphere of plants of this study was not merely due to it being abundant in the bulk soil that was used.

4.5 Limitations of experimental design

While some endophyte-associated effects were consistently shown across both replicates of the second experiment, the low sample size of only two replicates per endophyte treatment provided limited statistical power and meant that effects had to be very pronounced for statistical significance of differences to be shown. If a larger number of replicates were included, the statistical significance of subtler but potentially genuine effects may have been demonstrable. DNA extracts from the four replicates whose microbiomes were not analysed in this thesis have also been stored and are intended to be submitted for sequencing, which will then allow for effects observed in the second experiment to be assessed with the same statistical power as in the first experiment. At the same time however, given that these replicates did not grow as well as those that were analysed, the inclusion of these samples could also inadvertently add variability to the dataset. Given that next-generation sequencing platforms now allow for the multiplexing of hundreds of samples at continually increasing sequencing depths (Kozich et al., 2013), future studies should include a larger sample size than that used in this study to enable more statistically robust conclusions to be reached (Knight et al., 2012; Prosser, 2010).

Metabarcoding is a powerful technique that has enabled ground-breaking insights into the diversity and phylogenetic structure of complex microbial communities (Segata et al., 2013). However, there are a number of limitations intrinsic in the technique which should be taken into consideration when interpreting results. Given that it is a PCR-dependent technique, the choice of primers affects the resulting species profiles (see section 3.1.5). Prokaryotic and fungal copy number of rRNA genes can also differ widely between different prokaryotic and eukaryotic species (Bellemain et al., 2010; Větrovský & Baldrian, 2013),

and therefore the relative abundances of rRNA reads does not necessarily represent relative cellular abundances. Furthermore, DNA of dead cells is known to linger in soils for long periods of time, and this so-called 'relic' DNA can inflate diversity estimates (Carini et al., 2016). This caveat can be circumvented through such strategies as prior treatment of DNA samples with propidium monoazide (PMA) (Nocker et al., 2007) which binds to extracellular DNA inhibiting PCR amplification, thereby allowing for the selective amplification of DNA extracted solely from intact cells. Alternatively, the isolation and analysis of rRNA from RNA samples has the additional benefits of circumventing the detection of 'relic DNA', as well as enabling analysis of only those members of the microbiome that are physiologically active at the time of sampling (Turner et al., 2013). The storage of samples at -20^{o}C may have also introduced an additional bias, as it could have caused particular cells to lyse and for the DNA of such cells to potentially be degraded. However, while no published studies assessing fungal communities could be found, various storage conditions have been shown to have only minor impacts on bacterial community composition of soil samples (Lauber et al., 2010; Rubin et al., 2013).

4.6 Future directions

Despite it being well known that different host cultivar-endophyte strain symbioses result in unique biological outcomes for their hosts (Johnson et al., 2007), whether a similar extent of variability exists in the impacts of different endophyte strains on rhizosphere community composition of their hosts is unclear. Wakelin et al (2015) used denaturing gradient gel electrophoresis (DGGE) to assess the bacterial and fungal communities in the rhizosphere microbiome in response to two commercially widespread *E. festucae* var. *lolii* strains, and found evidence of subtle strain-specific effects of endophyte infection towards the rhizosphere microbiome. Similarly, Bell et al (2009) found that effects towards belowground bulk soil microbes were stronger in plants infected with wild-type endophyte than with the commercial strains AR1 (Fletcher, 1999) and AR37 (Popay & Wyatt, 1995). On the other hand, Rojas et al (2016) found that in *E. coenophiala*-infected tall-fescue differences in rhizosphere communities of plants infected with different endophyte strains did not differ as much compared with uninfected plants, suggesting there may not be a

high degree of strain specificity in endophyte-induced impacts on their hosts. This finding that endophyte strains with different alkaloid profiles do not differentially affect the rhizosphere microbiome also suggests that alkaloids do not play a role in the endophyte-associated effects observed in these studies. Future studies assessing the rhizosphere microbiome of a range of different plant cultivar-endophyte strain interactions are necessary in order to shed further light on the degree of variability in impacts of endophyte infection towards host rhizosphere community composition in different cultivar-endophyte strain interactions.

Studies have consistently shown that the factor that plays the strongest role in determining rhizosphere community composition is the soil biome in which plants are growing (Phillipot et al., 2013; Vandenkoornhuyse et al., 2015). Thus, it is of particular interest to identify effects associated with endophyte infection that are consistently observed in plants growing in a range of different soil types. This is also required to determine the extent to which the findings of single studies are representative of plants growing in other contexts. This could also reveal if a 'core microbiome' (Shade & Handelsman, 2012) exists in the roots of perennial ryegrass, potentially representing microbes more likely to be in direct interaction with their plant hosts rather than transient members that have stochastically colonized the rhizosphere purely due to their presence in the wider bulk soil. On the other hand it is also possible that there may not be a taxonomic core microbiome, and that instead a high level of functional redundancy may exist whereby different species from each soil biome are selected based on key functional traits rather than selecting for a particular taxonomic composition (Burke et al., 2011).

While this study focused solely on sampling the rhizosphere communities of plants, this is only one of a number of compartments which collectively comprise the complete plant microbiome. As outlined in the introduction (see section 1.1), the root microbiome is composed of three compartments- the rhizosphere, the rhizoplane (community living physically attached to the root), and the endosphere (community living inside the roots). Despite their close spatial proximity, each compartment has been shown to harbour compositionally distinct microbial communities (Chen et al., 2016; Edwards et al., 2015). Thus, despite our finding that the rhizosphere microbiome is unaffected by endophyte infection, studies that also sample the rhizoplane and endosphere microbiomes alongside

the rhizosphere will further our understanding of the effects that endophyte infection has on the complete root microbiome of their hosts. For instance, it could be argued that the endosphere and rhizoplane microbiomes are more likely to be affected by an endophyte-induced effect on the root transcriptome (Schmid et al., 2017) or metabolome (Wakelin et al., 2015), given their closer proximity to the root than the rhizosphere. Additionally, while the phyllosphere microbiome (microbial community associated with aboveground plant parts (Vorholt, 2012)) of perennial ryegrass in response to *Epichloë* infection has yet to be assessed using high-throughput sequencing, in tall-fescue it was found that the loline-secreting *Epichloë coenophiala* exerts selection pressure on the wider phyllosphere, as loline catabolizing *Burholderia* strains could be isolated from infected but not uninfected plants (Roberts & Lindow, 2014). *Epichloë festucae* var. *lolii* does not secrete lolines (Johnson et al., 2013), however the effects that endophyte infection has been shown to have towards the foliar metabolome (Rasmussen et al., 2008) could exert similar selection pressure towards the phyllosphere microbiome of their hosts.

Despite the results of this study suggesting that the community profile of the rhizosphere microbiome has relatively modest impacts on rhizosphere community composition, this does not necessarily mean that effects toward gene expression of the microbiome are as subtle. It has been suggested that assessing microbial communities at the functional level of genes using metatranscriptomics (Bashiardes et al., 2016) is more informative than at the level of species, given the ease with which microbes often share their genetic material (Burke et al., 2011). For example, in the rumen microbiome of sheep it was found that while taxonomic community composition remained largely unaffected between low and high methane-emitting sheep, alterations to the metatranscriptome were significantly associated with these differential states (Shi et al., 2014). Similarly, metatranscriptomic analysis of the rhizosphere microbiome of willows found that while community composition was largely indifferent between plants growing in contaminated versus non-contaminated soils, there was a comparatively larger difference between the metatranscriptomes of rhizospheres of plants growing in each soil (Yergeau et al., 2014). Future assessment of the rhizosphere metatranscriptome in response to endophyte infection will allow determination of whether endophyte infection impacts the collective gene expression of the rhizosphere microbiome of their hosts. As the costs of next-

generation sequencing continue to fall, such analyses are becoming increasingly affordable.

4.7 Conclusion

In conclusion, the results of this study show that in two independent experiments under controlled conditions growing in soil from a natural ryegrass pasture, *Epichloë festucae* var. *lolii* (Lp19) infection of perennial ryegrass (NuiD) had only a minor impact on the prokaryotic rhizosphere microbiome of their hosts. The overall composition of the fungal rhizosphere microbiome was also not significantly altered by endophyte infection, however an endophyte-associated antagonism towards a highly abundant OTU likely of the class Sordariomycetes was apparent in both experiments. While the statistical significance of this effect could not be shown in individual experiments, the effect was significant across both experiments. Future work implementing more targeted analyses are necessary to determine which particular species is represented by this OTU before the biological implications of this observed effect can be ascertained.

Due to the highly variable nature of both rhizosphere community composition as well as the biological outcomes of particular host cultivar-endophyte strain symbioses, the extent to which the findings of this study would also apply in other *Epichloë*-grass symbioses growing in different environmental contexts remains unclear. However, future analyses assessing a range of different host cultivar/endophyte strain symbioses will inevitably shed further light on this. While there is currently a clear focus towards utilizing high-throughput sequencing technologies to better understand the molecular basis underlying the symbiosis between *E. festucae* and *L. perenne* (Dupont et al., 2015; Eaton et al., 2010; Eaton et al., 2015; Schmid et al., 2017), this study represents a first step towards utilizing these powerful technologies to better understand the effects of endophyte infection towards the microbial 'second genome' (Berendsen et al., 2012) of their holobiont (Rosenberg & Zilber-Rosenberg, 2016) hosts. Irrespective of endophyte infection, future high-throughput analyses of the microbiome of perennial ryegrass may also eventually give rise to novel microbial strategies of promoting the growth and productivity of this agriculturally pivotal pasture grass.

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Zhu, S., Vivanco, J. M., & Manter, D. K. (2016). Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. *Applied Soil Ecology, 107*, 324-333.

Appendices

A1 Immunoblot sheets

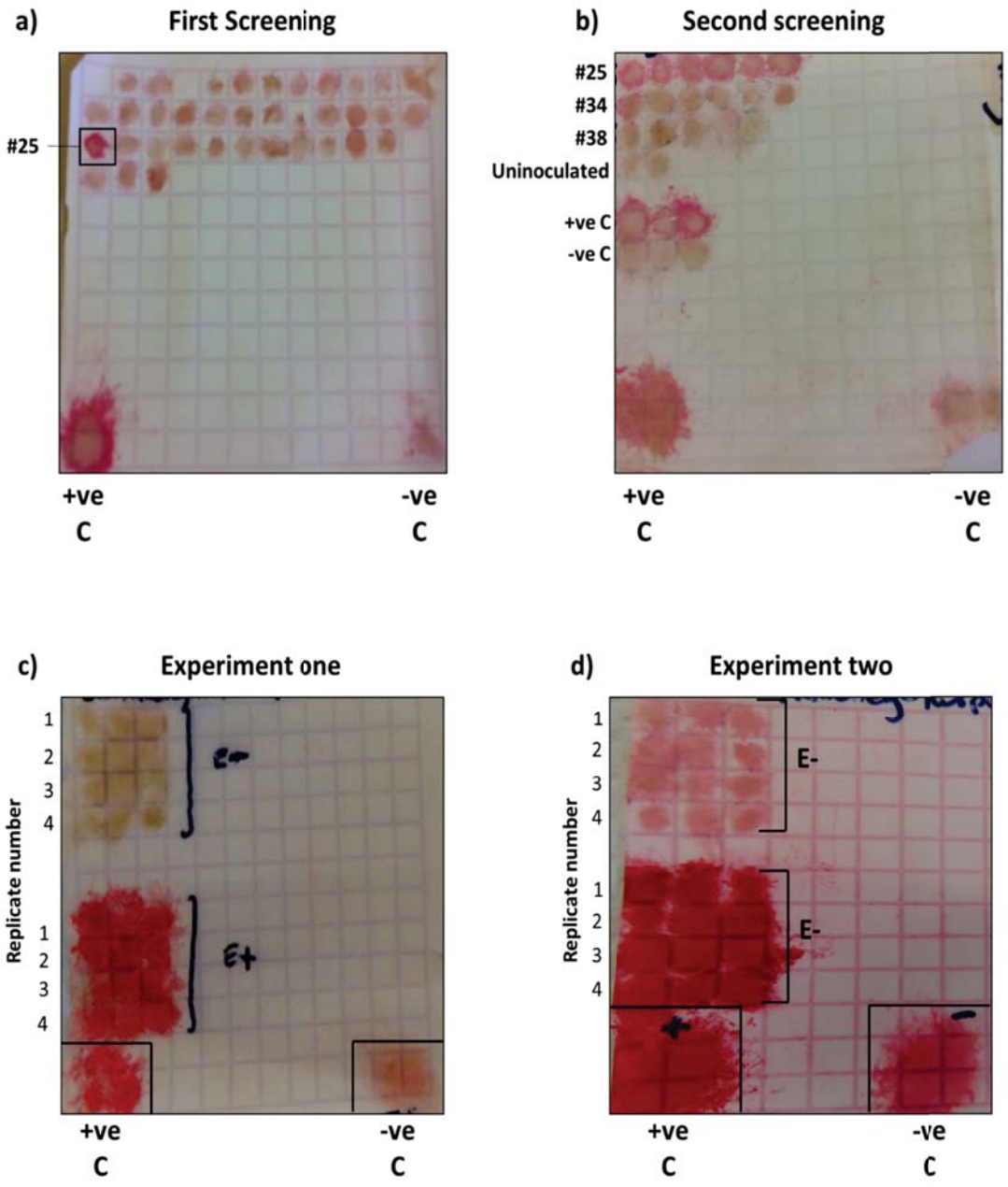

Figure A.1. Immunoblot results. a) Blot sheets of the first screening of inoculated plants **b)** Follow-up blot results confirming number 25 being successfully inoculated. **c, d)** Immunoblots carried out during each plant growth experiment to confirm infection status of replicates used. Three tillers were tested from each replicate.

A2 Physico-chemical characteristics of soil

Table A.1. Physico-chemical composition of soil used in each experiment.

Measure	Experiment one[2]	Experiment two[3]
pH	5.2	5.5
Olsen Phosphorus (mg/L)	109	97
K (me/100g)[1]	0.86	0.70
Ca (me/100g)[1]	7.6	7.7
Mg (me/100g)[1]	0.97	1.1
Na (me/100g)[1]	0.15	0.15
Cation Exchange Capacity (me/100g)[1]	21	21
Total base saturation	45	47
Volume weight	0.97	0.95

[1] me/100g= Milliequivalents per 100 g.

[2] Values are the average of three individual samples analysed.

[3] Values are the average of two individual samples analysed.

A3 *Bacillus thuringiensis* treatment in experiment two did not increase relative abundances of *Bacillus* OTUs in rhizosphere or bulk soil samples

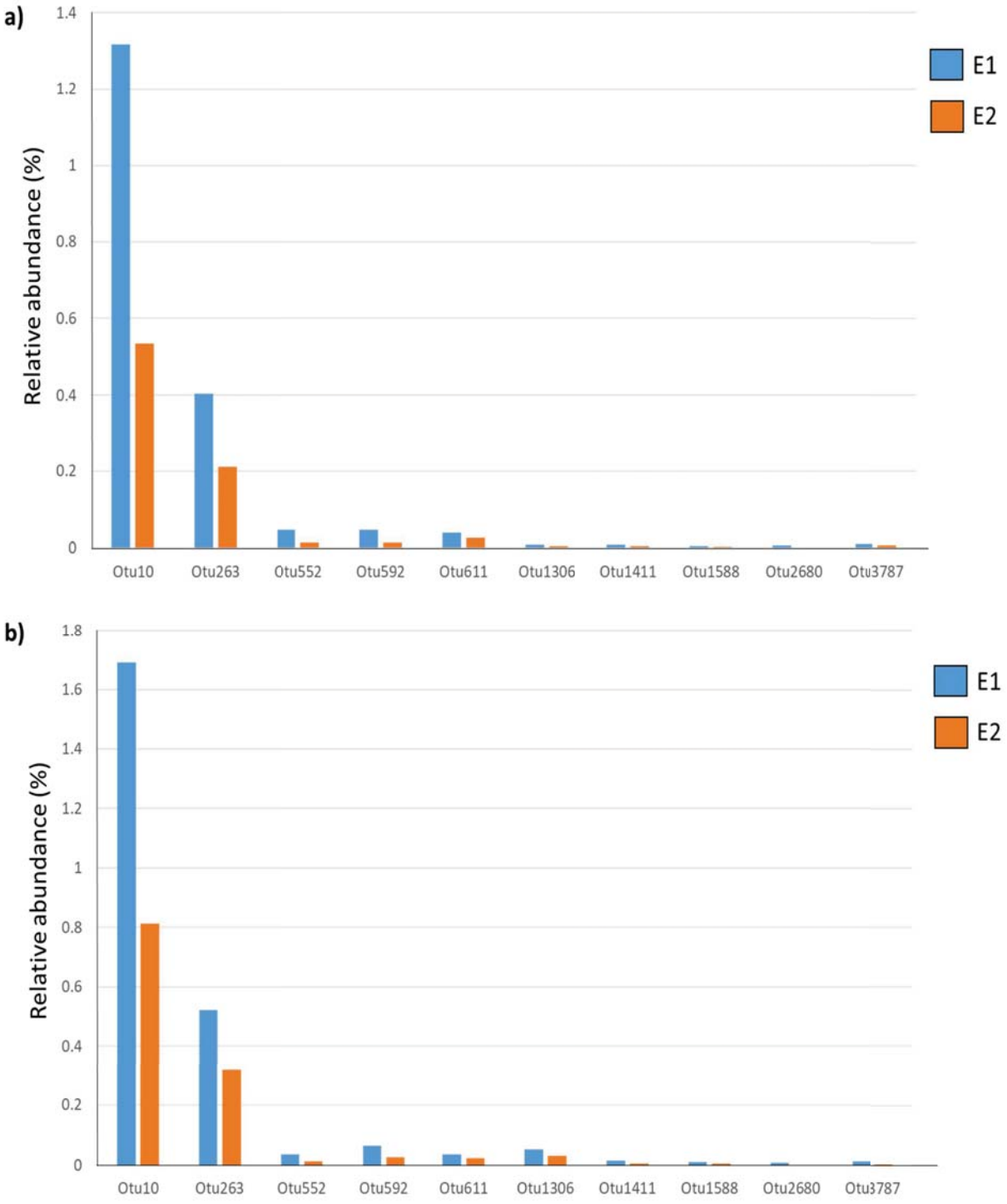

Figure A.2. Average abundances of OTUs assigned to the genus *Bacillus* between experiments. Rhizosphere and **b)** Bulk-soil. E1/E2= Experiment one/two

A4 Concentrations of DNA extracts

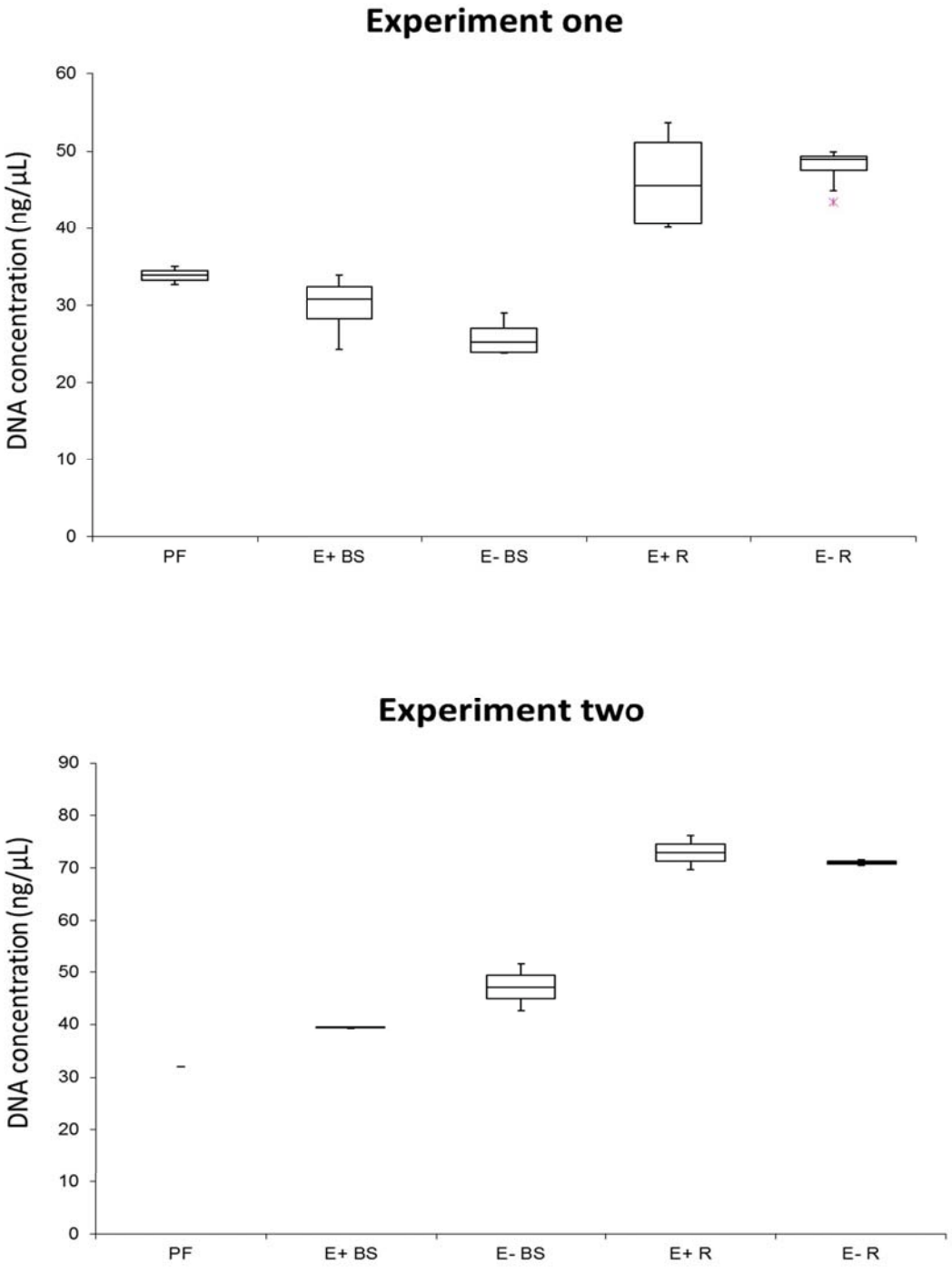

Figure A.3. Concentrations of DNA extracts. All extractions were carried out using the Powersoil DNA isolation kit (see methods), and were eluted in 100 µL. PF=Plant-free, E+/-= Endophyte-positive/negative, BS=Bulk soil, R= Rhizosphere.

A5 Numbers of reads and OTUs in unrarefied and rarefied OTU tables

Table A.2. Number of reads, OTUs, Chao1 richness estimates and estimated OTU coverage rates in unrarefied samples.

		Prokaryotes				Fungi			
Experiment	Sample name[1]	Number of reads	Number of OTUs	Chao1 estimate	Estimated Coverage (%)	Number of reads	Number of OTUs	Chao1 estimate	Estimated coverage (%)
1	PF1	43123	2507	3030	82.7	44229	1385	1664	83.3
	PF2	60827	2668	3072	86.9	33161	1373	1679	81.8
	-1B	40722	2478	2999	82.6	55192	1680	1948	86.3
	-2B	50713	2624	3096	84.8	46423	1530	1804	84.8
	-3B	30790	2340	2973	78.7	17802	1120	1580	70.9
	-4B	50019	2636	3114	84.6	48045	1453	1735	83.8
	-1R	34153	2610	3235	80.7	29087	1203	1659	72.5
	-2R	35433	2672	3344	79.9	34903	1399	1772	78.9
	-3R	48862	2854	3396	84	55697	1498	1814	82.6
	-4R	25462	2395	3080	77.8	31385	1038	1489	69.7
	+1B	23976	2214	2874	77	49708	1580	1816	87
	+2B	72060	2816	3213	87.6	58762	1577	1862	84.7
	+3B	70489	2768	3164	87.5	57367	1479	1636	90.4
	+4B	73542	2958	3376	87.6	42391	1412	1697	83.2
	+1R	49513	2826	3282	86.1	52398	1407	1580	89.0
	+2R	79463	3168	3518	90	86748	1781	2060	86.5
	+3R	42748	2832	3293	86	48810	1348	1708	78.9
	+4R	62529	2999	3381	88.7	43574	1302	1716	75.9
2	PF1	32657	2262	2863	79	38825	1399	1626	86
	-1B	30251	2182	2795	78	49834	1495	1721	86.9
	-3B	26932	2119	2730	77.6	41189	1141	1469	77.7
	-1R	22838	2246	2901	77.4	41939	1284	1677	76.6
	-3R	34403	2361	2901	80.1	75192	1263	1575	80.2
	+2B	45887	2413	2823	85.4	48870	1380	1654	83.4
	+4B	35052	2278	2768	82.3	39448	1304	1546	84.3
	+2R	44832	2477	2957	83.8	67716	1291	1538	84
	+4R	30784	2304	2824	81.6	39070	1064	1399	76.1

[1] PF= Plant-free; B= Bulk-soil; R= Rhizosphere +/- Endophyte-infected/uninfected. Numbers in each plant containing sample name denote the replicate number.

Table A.3. Number of reads, OTUs, Chao1 richness estimates and estimated OTU coverage rates of rarefied samples.

Experiment	Sample name[1]	Prokaryotes				Fungi			
		Number of reads	Number of OTUs	Chao1 estimate (unrarefied)	Estimated Coverage (%)	Number of reads	Number of OTUs	Chao1 estimate (unrarefied)	Estimated Coverage (%)
1	PF1	22838	2158	3030	71.2	17802	1035	1664	62.2
	PF2	22838	2069	3072	67.4	17802	1128	1679	67.2
	-1B	22838	2080	2999	69.4	17802	1262	1948	64.8
	-2B	22838	2131	3096	68.8	17802	1158	1804	64.2
	-3B	22838	2135	2973	71.8	17802	1120	1580	70.9
	-4B	22838	2139	3114	68.7	17802	1078	1735	62.1
	-1R	22838	2344	3235	72.5	17802	987	1659	59.5
	-2R	22838	2363	3344	70.7	17802	1117	1772	63
	-3R	22838	2337	3396	68.8	17802	1014	1814	55.9
	-4R	22838	2336	3080	75.8	17802	827	1489	55.5
	+1B	22838	2190	2874	76.2	17802	1199	1816	66
	+2B	22838	2112	3213	65.8	17802	1138	1862	61.1
	+3B	22838	2124	3164	67.1	17802	1098	1636	67.1
	+4B	22838	2180	3376	64.6	17802	1084	1697	63.9
	+1R	22838	2350	3282	71.6	17802	1055	1580	66.8
	+2R	22838	2406	3518	68.4	17802	1076	2060	52.2
	+3R	22838	2407	3293	73.1	17802	917	1708	53.7
	+4R	22838	2317	3381	68.6	17802	927	1716	54
2	PF1	22838	2039	2863	71.2	17802	1114	1626	68.5
	-1B	22838	2037	2795	72.9	17802	1156	1721	67.2
	-3B	22838	2027	2730	74.2	17802	864	1469	58.8
	-1R	22838	2246	2901	77.4	17802	954	1677	56.9
	-3R	22838	2137	2901	73.7	17802	761	1575	48.3
	+2B	22838	1987	2823	70.4	17802	1138	1654	68.8
	+4B	22838	2012	2768	72.7	17802	1034	1546	66.9
	+2R	22838	2044	2957	69.1	17802	804	1538	52.3
	+4R	22838	2126	2824	75.3	17802	819	1399	58.5

[1] PF= Plant-free; B= Bulk-soil; R= Rhizosphere +/- Endophyte-infected/uninfected. Numbers in each plant containing sample name denote the replicate number.

A6 Rarefaction curves of prokaryotic and fungal samples rarefied to 70,000 reads per sample

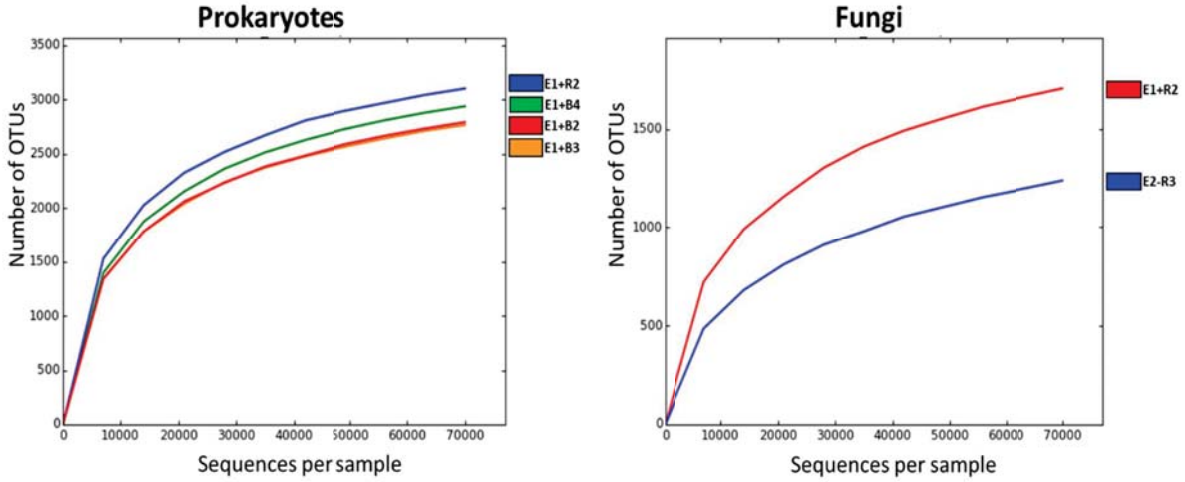

Figure A.4. Rarefaction curves of species richness at 70,000 reads per sample. Only the few prokaryotic and fungal samples containing enough reads to rarefy at this depth could be included. E1/E2= Experiment one/two; +/-= endophyte-infected/uninfected; B/R= Bulk-Soil/Rhizosphere. Numbers at the end of each sample name correspond to the replicate number.

A7 Figure 18, prokaryotic phyla legend

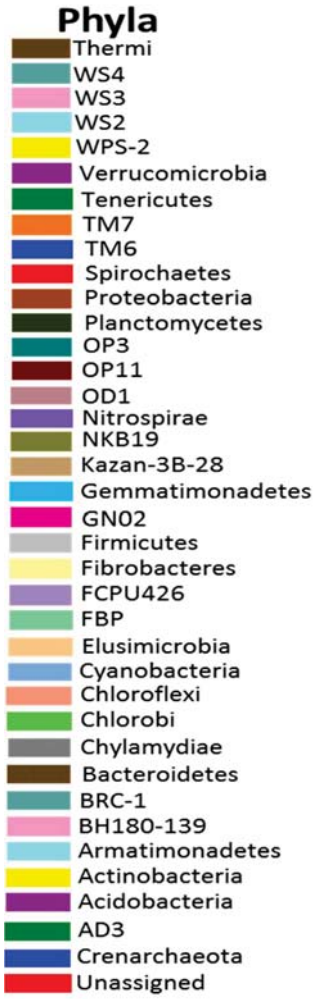

Figure A.5. Full legend for Figure 18 a containing all prokaryotic phyla. Phyla are listed in the same order in which they appear on the plots.

A8 OTU1 representative sequence and BLAST results

a) >Otu1
TAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTAT
GGAGTCTCTAACTCCTAAACCATTTGTGAACCTAATTTTACCATTGTTTCGGCAGGTTGCT
GCCAGTCTCTATTGTGACTGTCGGTAGCCTGTCGGATGTGCTTAAAACCCTGGTTGTTGG
TGTGTAGATTCTCTGAGTCTCAAAAACAAGTCAAAACTTTCAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAA

b) **Query 1 of 1:** Otu1

	Reference	Score	E-value	Prcnt	MisM	Qstart	Qend	Rstart	Rend
*	KC222743 **SH176264.07FU** Sordariales	326	7e-88	89.92	0018	13	266	1	254
*	GU187831 **SH176264.07FU** Sordariomycetes	326	7e-88	89.92	0018	13	266	1	254
*	HQ829350 **SH176264.07FU** Sordariomycetes	320	3e-86	89.53	0019	13	266	1	254
	AY821864 **SH200152.07FU** Ceratocystis paradoxa (as Thielav..	147	6e-34	89.74	0009	1	116	12	126
	KY310660 **SH200140.07FU** Ceratocystis ethacetica (as Thiel..	145	2e-33	95.56	0004	1	90	4	93
	KX954597 **SH200140.07FU** Ceratocystis ethacetica (as Thiel..	145	2e-33	95.56	0004	1	90	11	100
	KX954596 **SH200140.07FU** Thielaviopsis musarum	145	2e-33	95.56	0004	1	90	11	100
	KX954595 Thielaviopsis musarum	145	2e-33	95.56	0004	1	90	11	100
	KR183793 **SH200140.07FU** Ceratocystis ethacetica (as Thiel..	145	2e-33	95.56	0004	1	90	13	102
	KM519456 **SH200140.07FU** Ceratocystis paradoxa (as Thielav..	145	2e-33	95.56	0004	1	90	1	90
	KF977129 Fungi	145	2e-33	95.60	0002	1	90	235	146
	KC305159 **SH200140.07FU** Ceratocystis paradoxa (as Thielav..	145	2e-33	95.56	0004	1	90	2	91
	JQ717359 **SH203376.07FU** Sarocladium strictum	145	2e-33	95.60	0002	1	90	13	102
	FJ808063 Ceratocystis	145	2e-33	95.56	0004	1	90	11	100
	FJ808043 Ceratocystis	145	2e-33	95.56	0004	1	90	11	100
	DQ318203 **SH200140.07FU** Ceratocystis paradoxa (as Thielav..	145	2e-33	95.56	0004	1	90	260	349
	KU978914 **SH200146.07FU** Ceratocystis radicicola (as Thiel..	143	7e-33	96.51	0003	5	90	5	90
	KU978911 **SH200146.07FU** Ceratocystis radicicola (as Thiel..	143	7e-33	96.51	0003	5	90	2	87
	JN396503 Fungi	143	7e-33	77.94	0031	1	263	90	341
	JN396425 Fungi	143	7e-33	77.94	0031	1	263	90	341
	GQ514912 Fungi	143	7e-33	78.23	0025	1	263	11	255
	GQ511931 Fungi	143	7e-33	78.23	0025	1	263	11	255
	KR183792 **SH200140.07FU** Ceratocystis paradoxa (as Thielav..	141	3e-32	95.45	0004	3	90	17	104
	KX954598 **SH200141.07FU** Ceratocystis paradoxa (as Chalaro..	139	1e-31	94.44	0005	1	90	11	100
	KT963173 **SH200141.07FU** Ceratocystis paradoxa (as Chalaro..	139	1e-31	94.44	0005	1	90	9	98
	KT963170 **SH200141.07FU** Ceratocystis paradoxa (as Chalaro..	139	1e-31	94.44	0005	1	90	9	98
	KT963166 **SH200141.07FU** Ceratocystis paradoxa (as Chalaro..	139	1e-31	94.44	0005	1	90	2	91
	KP133190 **SH219560.07FU** Cephalosporium	139	1e-31	89.47	0007	1	112	12	122
	KJ956786 **SH200142.07FU** Thielaviopsis basicola	139	1e-31	94.44	0005	1	90	69	158
	KJ511486 **SH200141.07FU** Ceratocystis paradoxa (as Chalaro..	139	1e-31	94.44	0005	1	90	1	90

c)

	Description	Max score	Total score	Query cover	E value	Ident	Accession
*	Uncultured fungus clone HFEsoil59 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S rib	326	326	95%	2e-85	90%	KC222743.1
*	Uncultured fungus clone RFLP10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribos	326	326	95%	2e-85	90%	GU187831.1
*	Uncultured fungus clone RFLP36 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribos	320	320	95%	1e-83	90%	HQ829350.1
	Uncultured Ascomycota clone 736 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribo	174	174	100%	9e-40	79%	HM162169.1
✓	Xylariales sp. 4-16 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ger	169	169	100%	4e-38	79%	HQ316570.1
✓	Xylariales sp. 4-4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene	169	169	100%	4e-38	79%	HQ316561.1
✓	Xylariales sp. LM40 18S ribosomal RNA gene, partial sequence	169	169	100%	4e-38	79%	EF060424.1
✓	Gaeumannomyces incrustans I3 18S, 5.8S and 26S rRNA genes, partial sequence	169	169	100%	4e-38	79%	U17215.1
✓	Gaeumannomyces incrustans I1 18S and 5.8S rRNA genes, partial sequence	169	169	100%	4e-38	79%	U17214.1
✓	Phialemonium sp. strain Hulcr5398 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S rib	167	167	100%	1e-37	79%	KU961666.1
	Uncultured fungus clone 034A1015 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	167	167	100%	1e-37	79%	JX318747.1
✓	Magnaporthales sp. P91-379 18S ribosomal RNA gene, partial sequence	167	167	98%	1e-37	79%	KJ439177.1
✓	Magnaporthales sp. P90-52 18S ribosomal RNA gene, partial sequence	167	167	98%	1e-37	79%	KJ439176.1
✓	Nectria lamyi strain CBS 127385 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribos	165	165	100%	5e-37	78%	HM534898.1
✓	Gaeumannomyces incrustans isolate M51 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and ;	163	163	98%	2e-36	79%	JF414846.1
	Uncultured fungus clone 037A33776 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	161	161	100%	7e-36	78%	JX350860.1
	Uncultured fungus clone 037A30638 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	161	161	100%	7e-36	78%	JX347759.1
	Uncultured fungus clone 034A4171 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	161	161	100%	7e-36	78%	JX321853.1
✓	Fungal sp. ARIZ L365 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA	161	161	98%	7e-36	79%	FJ612791.1
✓	Thyronectria pinicola strain 201406bj073 18S ribosonal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 2	158	158	100%	9e-35	78%	KU886299.1
✓	Magnaporthaceae sp. isolate CSB_F400 18S ribosonal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 2	158	158	99%	9e-35	78%	KU680396.1
✓	Thyronectria pinicola voucher DAR80240 18S ribosonal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 2	158	158	100%	9e-35	78%	KP751375.1
	Uncultured fungus clone 107A62770 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	156	156	100%	3e-34	78%	JX328957.1
	Uncultured fungus clone 034A4296 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	156	156	100%	3e-34	78%	JX321975.1
✓	Magnaporthales sp. P45-527 18S ribosomal RNA gene, partial sequence	156	156	98%	3e-34	78%	KJ439144.1

Figure A.6. Representative sequence of OTU 1 and results of BLAST alignments. a) Representative sequence of OTU 1. b) BLAST search results using the UNITE database. c) BLAST search results using the 'nr' BLAST database. the top three hits in both databases (asterisks) corresponded to the same three reference sequences. Ticked boxes in c) represent hits to reference sequences assigned as belonging to the class Sordariomycetes.

A9 Significantly differentially abundant OTUs between rhizospheres of plants of each experiment

Table A. 4. Significantly differentially abundant prokaryotic OTUs in rhizospheres of plants between each experiment.

OTU id	Base mean[1]	Log2fold change[2]	P-value[3]	Predicted taxonomy[4]
15	113.1	6.5	1.7e-26	Firmicutes; g Alicyclobacillus
34	77.8	6.0	7.3e-23	Chloroflexi; c Ellin6529
52	53.4	5.4	1.1e-16	Verrucomicrobia; o Chthoniobacterales
118	50.6	5.4	3.7e-17	Acidobacteria; o Acidobacteriales
153	45.8	5.2	2.1e-16	Acidobacteria; o Ellin6513
46	46	5.2	2.9e-16	WPS-2
171	43.1	5.1	2e-15	Proteobacteria; o SC-I-84
181	39.4	5.0	1e-14	Proteobacteria; Rhizobiales
125	38.8	5.0	3.9e-14	Proteobacteria; o Xanthomonadales
82	37.2	4.9	6e-14	Acidobacteria; o Acidobacteriales
57	37.7	4.9	1.2e-13	Verrucomicrobia; o Chthoniobacteriales
73	36.1	4.9	1.2e-13	Proteobacteria; o Xanthomonadales
119	36.3	4.8	1.5e-12	Acidobacteria; o DS-18
107	34.6	4.8	2.5e-13	Proteobacteria; o Ellin329
61	34.3	4.8	1.9e-12	Acidobacteria; o Acidobacteriales
452	32.9	4.8	1.2e-12	Proteobacteria; o Sphingomonadales
446	33.7	4.8	3.4e-12	Chloroflexi; c Ellin6529
195	44.2	4.7	5.7e-8	Cyanobacteria; o Strameopiles
199	29.9	4.6	1.5e-11	Acidobacteria; f Ellin6075
322	28	4.5	2.4e-11	Proteobacteria; o Rhodospirillales
99	26.6	4.5	8.5e-11	Proteobacteria; o Rhorospirillales
330	31.3	4.3	5.8e-7	Proteobacteria; o Myxococcales
576	39.2	4.3	6.2e-6	Proteobacteria; o Burkholderiales
311	29.2	4.2	9.9e-7	Proteobacteria; o Myxococcales
135	29.1	4.2	1.3e-6	Acidobacteria; o Ellin6513
97	27.6	4.1	3.1e-6	Proteobacteria; o Sphingomonadales
127	22.9	3.9	7.3e-6	Proteobacteria; o Rhodospirillales
287	22.2	3.9	5.7e-6	Bacteroidetes; f Chitinophagaceae
475	23.3	3.7	1.1e-4	Verrucomicrobia; f Opitutaceae
159	22.1	3.7	1.4e-4	Proteobacteria; o Xanthomonadales
166	20.7	3.6	2.2e-4	Proteobacteria; c Betaproteobacteria
140	19.9	3.5	2.5e-4	Proteobacteria; o Myxococcales
186	20.2	3.5	2.8e-4	Proteobacteria; o Ellin329
24	284.9	-3.2	1.1e-8	Proteobacteria; f Burkholderiaceae
70	71.5	2.9	5.3e-6	Proteobacteria; o Xanthomonadales
49	60.8	-2.8	0.01	Actinobacteria; o Actinomycetales
129	61.3	-2.7	0.01	Actinobacteria; f Microbacteriaceae
85	77.9	-2.6	0.02	Proteobacteria; Burkholderiales
40	48.3	-2.6	0.01	Actinobacteria; o Actinomycetales
142	120.9	-2.5	7.7e-7	Actinobacteria; f Gaiellaceae
112	81.6	-2.4	0.01	Actinobacteria; o Actinomycetales
981	49.6	-2.3	0.03	Actinobacteria; o Frankiaceae
314	65.5	-2.3	0.02	Actinobacteria; f frankiaceae
106	35.1	-2.2	0.04	Actinobacteria; o Actinomycetales
164	44.5	2.2	0.005	Proteobacteria; f Rhodospirillaceae
32	47.6	-2.0	0.046	Actinobacteria; f Gaiellaceae
158	86.1	-2.0	0.001	Verrucomicrobia; f Pedosphaerales

8	205.6	-1.9	1.1e-16	Actinobacteria; o Actinomycetales
27	60.8	-1.9	0.03	Actinobacteria; o Actinomycetales
402	73	-1.7	0.04	Bacteroidetes; o Sphingobacteriales
20	71.6	-1.7	1.8e-4	Actinobacteria; f Gaiellaceae
72	167	1.6	1.1e-24	Verrucomicrobia; f Chthoniobacteraceae
103	52.6	-1.6	0.049	Proteobacteria; o Xanthomonadales
211	63.1	-1.6	0.03	Proteobacteria; o Myxococcales
104	104.5	-1.5	9.9e-15	Proteobacteria; f Burkholderaceae
88	86.2	-1.4	2e-11	Actinobacteria; f Gaiellaceae
37	170.8	-1.4	9.8e-18	Actinobacteria; o Actinomycetales
76	106.3	-1.4	3.6e-8	Actinobacteria; Solirubrobacterales
196	165.6	-1.4	5.7e-8	Proteobacteria; -o Burkholderiales
18	153.5	-1.4	3.3e-26	Actinobacteria; f Gaiellaceae
51	68.5	-1.3	2.4e-4	Acidobacteria ; o iii1-15
344	59.2	-1.2	0.048	Proteobacteria; o Sphingomonadales
133	188.4	-1.2	1.6e-7	Proteobacteria; o Burkholderiales
30	74.7	1.2	0.008	Proteobacteria; o Rhizobiales
156	86.6	-1.1	2.2e-6	Actinobacteria; o Solirubrobacterales
60	82.6	-1.1	1.1e-6	Actinobacteria; o Solirubrobacterales
22	158.3	-1.1	5.5e-16	Proteobacteria; o SC-I-84
63	62.3	-1.1	3e-5	Verrucomicrobia; o Chrhoniobacterales
113	59	-1.0	0.0003	Acidobacteria; o iii1-15
55	70.8	-1.0	1.6e-7	Actinobacteria; o Gaiellaceae
44	104.7	-1.0	0.0003	Actinobacteria; o Solirubrobacterales
147	71.2	-0.9	0.0002	Actinobacteria; o Actinomycetales
54	109.7	-0.9	1e-09	Acidobacteria; o Acidobacteriales
200	116	-0.9	0.0003	Verrucomicrobia; o Cthoniobacterales
848	68	-0.8	0.004	Bacteroidetes; o Sphingobacteriales
109	194.5	-0.8	1.5e-13	Acidobacteria; o iii1-15
283	58.5	-0.8	0.004	Planctomycetes; f Isosphaeraceae
42	74.2	-0.8	0.0002	Planctomycetes; o WD2101
190	117.6	-0.8	0.0001	Acidobacteria; o Acidobacteriales
43	160.5	0.7	1.3e-6	Acidobacteria; o 32-20
86	120.9	-0.7	7.7e-7	Actinobacteria; f Gaiellaceae
6	279.8	0.7	3.9e-7	Crenarchaeota; o Nitrososphaerales
11	213	-0.7	0.003	Actinobacteria; o Solirubrobacterales
84	73	-0.7	0.0005	Actinobacteria; o Actinomycetales
12	129.3	-0.6	4.6e-5	Acidobacteria; f Ellin6075
53	351	-0.6	0.007	Proteobacteria; o Burkholderiales
318	75.8	-0.6	0.007	Actinobacteria; o Solirubrobacterales
71	99.7	-0.6	1.7e-5	Proteobacteria; o Rhizobiales
121	77.7	-0.6	0.004	Acidobacteria; o Acidobacteriales
105	73.4	-0.6	0.047	Proteobacteria; o Xanthomonadales
75	97	-0.6	0.0005	Chloroflexi; c Ellin6529
35	110.4	-0.6	0.0006	Proteobacteria; o Xanthomonadales
23	146.1	-0.6	2.5e-5	Chloroflexi; c Ellin6529
25	211.5	0.5	0.0002	Acidobacteria; o Acidobacteriales
120	79.5	0.5	0.004	Acidobacteria; o Acidobacteriales
256	61.2	-0.5	0.01	Proteobacteria; o Xanthomonadales
36	211.5	-0.5	0.0001	Acidobacteria; o iii1-15
39	278	-0.5	0.04	Proteobacteria; o Sphingomonadales
117	117.4	0.5	0.01	Bacteroidetes; Sphingobacteriales
152	109.2	0.5	0.0005	Acidobacteria; o Acidobacteriales
19	161.4	-0.5	6.9e-5	Chloroflexi;c Ellin6529
637	68.3	0.5	0.03	Proteobacteria; o Rhizobiales
16	213.7	0.5	0.0001	Proteobacteria; o Xanthomonadales
94	104.2	-0.4	0.049	Proteobacteria; o Xanthomonadales

4	247.2	-0.4	0.004	Crenarchaeota; o Nitrososphaerales
9	215.5	0.4	0.004	Proteobacteria; f EB1003
1245	106.7	-0.4	0.03	Proteobacteria; f EB1003
2	600.8	-0.4	0.001	Verrucomicrobia; o Cthoniobacterales
28	114.2	0.3	0.048	Proteobacteria; o Rhizobiales
7	456	-0.3	0.01	Proteobacteria; o Rhizobiales
10	403.2	0.2	0.03	Firmicutes ; g Bacillus
1	1684.3	0.2	0.02	Firmicutes; o Bacillales

[1] The average number of reads of that OTU across all samples of each comparison.

[2] Positive values (black) denote higher in E1 than E2, negative values (red) denote higher in E2 than E1.

[3] False Discovery rate (FDR)-corrected.

[4] Assigned Phyla followed by the highest level for which each OTU was assigned taxonomy.

Table A. 5. Significantly differentially abundant fungal OTUs in rhizospheres of plants between each experiment.

OTU id	Base mean[1]	Log2fold change[2]	P-value[3]	Predicted taxonomy[4]
25	150.1	7.4	7.4e-21	Unidentified
43	98.4	6.7	1.3e-14	Ascomycota; g Candida
79	83.3	6.6	2.3e-18	Unidentified
64	80.8	6.5	1e-17	Ascomycota; f Trichocomaceae
49	72.7	6.4	1.1e-16	Unidentified
95	62.6	6.1	1.2e-14	Unidentified
72	63.7	5.9	1.3e-6	Ascomycota; f Incertae sedis
86	44.1	5.6	4.1e-13	Unidentified
82	38.1	5.4	1.4e-11	Zygomycota; o Mucorales
80	36.1	5.3	3.7e-8	Ascomycota; Unidentified
169	31.8	5.2	2.7e-11	Unidentified
14	1208.9	-5.1	2.8e-5	Unidentified
108	34.3	5.1	2.6e-5	Unidentified
159	33.3	5.0	2.8e-5	Unidentified
63	25.9	4.8	1.4e-7	Ascomycota; f Hypocreaceae
201	25.3	4.7	5e-5	Unidentified
58	24.3	4.6	5.8e-5	Unidentified
67	21.6	4.5	5.3e-5	Basidiomycota; f Marasmiaceae
89	18.9	4.3	1.1e-4	Ascomycota; o Hypocreales
24	130.0	3.6	2e-5	Ascomycota; f Trichocomaceae
19	1092.2	-3.0	4.8e-11	Unidentified
60	154.6	-3.0	3.2e-4	Zygomycota; f Moertierellaceae
126	83.9	-2.8	0.035	Ascomycota; f Pyronemataceae
11	276.3	-2.8	2.3e-14	Unidentified
59	109.2	-2.6	0.02	Unidentified
36	252.4	-2.3	2.6e-8	Unidentified
8	359.2	-2.2	3.2e-15	Ascomycota; f Nectriaceae
122	48.9	2.1	0.044	Unidentified
20	134.5	-2.0	4e-8	Unidentified
22	93.5	-2.0	0.035	Unidentified
13	351.6	-1.9	3.7e-8	Basidiomycota; o Filobasidiales
450	60.4	-1.8	0.046	Ascomycota; Unidentified
163	164.6	-1.8	1e-5	Ascomycota; Unidentified
2	3244.6	-1.7	9.9e-7	Ascomycota; f Nectriaceae
35	128.6	-1.7	0.003	Ascomycota; f Hypocreaceae
18	292.3	-1.7	1.1e-4	Ascomycota; f Clavicipitaceae
32	138.2	-1.5	3.4e-7	Unidentified
10	260.7	-1.4	0.01	Unidentified
33	103.6	-1.3	2.4e-4	Unidentified
41	180.6	-1.1	0.02	Unidentified
30	160.2	0.9	0.01	Unidentified
5	2072.2	-0.9	0.002	Unidentified
31	114.5	-0.9	0.035	Ascomycota; f Lasiophaeriaceae
12	305.1	-0.8	0.009	Zygomycota; f Mortierellaceae
6	644.7	-0.7	0.035	Ascomycota; f Sporomiaceae

[1] The average number of reads of that OTU across all samples of each comparison.

[2] Positive values (black) denote higher in E1 than E2, negative values (red) denote higher in E2 than E1.

[3] False discovery rate (FDR)-corrected.

[4] Assigned phyla followed by the highest level for which each OTU was assigned taxonomy to the genus level.