# Title: Variation in bacterial species composition of the rhizosphere microbiome is linked to variation in plant root phenotypes and fitness, according to competitive environment belowground

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Variation in bacterial species composition in the rhizosphere microbiome of *Ipomoea purpurea* is linked to variation in plant root phenotypes and fitness, according to competitive environment belowground

## Abstract

Premise

Understanding if and how root phenotypes and the rhizosphere microbiome can influence each other and alter plant responses to belowground competition remains an important and elusive challenge in evolutionary ecology. To address this gap we asked, Does the rhizosphere bacterial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment?

Methods

We analyzed rhizosphere soil samples taken from our focal species, *Ipomoea purpurea,* that was grown in the presence and absence of competition with a closely related competitor, *I. hederacea,* in a randomized field experiment.

Results

We found evidence for linear associations between root traits and the rhizosphere microbiome. Further, our work uncovered a significant interaction effect between competitive environment and bacterial species richness on plant fitness. Specifically, we found that an increase in bacterial richness was associated with an increase in plant fitness when plants are grown in the presence of competition but found no evidence of a relationship in the absence of competition.

Conclusions

Our results indicate that rhizosphere bacterial richness may have a direct and positive impact on how *I. purpurea* competes for belowground competition. We discuss the ecological and evolutionary implications of our results and how future work can help uncover the underlying mechanisms behind our findings.

**Key words: Ipomoea; rhizosphere; microbiome; alpha-diversity; beta-diversity**

## Introduction

A major and unresolved challenge in plant evolutionary ecology is understanding the relative role that plant-microbe interactions may play in the feedbacks between plant ecology and evolution. Evidence is accumulating that plant community structure and composition are driven by complex interactions between plant functional traits, the associated microbial communities of plants and environmental conditions (*e.g.,* soil quality, nutrient stress, or competitive interactions; Reynolds et al. 2014; Bever et al. 2012; Bardgett et al. 2014; Fitzpatrick et al. 2018). Recent research has indicated that belowground root traits may play an important role in shaping the root microbiome (Saleem et al. 2018), by significantly altering soil biophysical and edaphic properties (*e.g.,* aggregation, structure, pH and moisture). As a result, variation in root traits may promote variation in rhizosphere community structure and phenotypic differences in root traits may potentially lead to greater differentiation in the rhizosphere community between plants, *i.e.,* influence community composition. Further, recent research demonstrates that interactions between plant phenotypes and the microbial community can alter plant fitness linked traits (*e.g.,* flowering phenology) and therefore potentially alter plant evolution (Lau and Lennon 2011; Wagner et al. 2014; Panke-Buisse et al. 2015; *discussed in* Rebolleda‐Gómez et al. 2019; Chaney and Baucom 2020). Despite the ecological and evolutionary implications of root-microbe interactions for plant systems, much of the published work examines root phenotypes of crop species (Roeland et al. 2012) and does not consider the interaction of root phenotypes and plant-plant competition, an important and ubiquitous agent of plant stress. Furthermore, evidence demonstrating that plant phenotypes can influence the root microbiome, and research reconciling the additive and synergistic effects of root phenotypes and the soil microbial community to feedback into belowground plant-plant competition (belowground competition hereafter) are lacking.

Due to its primary function in acquiring essential nutrients and water from the soil environment, a plant’s root system plays a pivotal role in mediating competition for limiting resources belowground. The root system is a complex multicellular organ composed of many traits that can be broadly classified into four functional groups including traits that capture the spatial distribution of the root system, or root architecture (*e.g.,* angle formed between roots, root width and length) and traits that describe specific characteristics of individual roots, or root morphology (*e.g.,* root diameter, lateral root number). The root system can also be more coarsely described based on its overall size, including traits such as root surface area and/or biomass/volume and general shape, or topology (*e.g.,* root system width with soil depth). Because traits linked to each of these functional groups tend to behave in an integrated manner, their accumulated effects can therefore impact the resources that are readily available to plants and the extent to which plants can exploit and compete for them (*e.g.,* uptake efficiency; Lynch 1995; York et al. 2013). In recent work, we demonstrated that belowground competition can potentially influence the evolution of root traits and therefore may play an overlooked role in driving patterns of plant diversity and distributions (Colom and Baucom, 2020; Colom and Baucom In Prep). In our past work, however, we did not consider whether the microbial community in the root-soil interface (rhizosphere microbiome hereafter) was associated with root phenotypes nor did we test for evidence that the rhizosphere microbiome could alter plant fitness according to competitive environment.

The rhizosphere microbiome may influence plant function via facilitating plant nutrient uptake, stimulating plant growth, increasing tolerance to stressful environments, and protecting against pathogens (Grichko and Glick 2001; Mayak et al. 2004; van der Heijden et al. 2008; Upadhyay et al. 2009; Verbon and Liberman, 2016; Jacoby et al. 2017; Kwak et al. 2018). In addition, the rhizosphere microbiome can also elicit phenotypic plasticity of root traits, potentially influencing root function (*discussed in* Friesen et al. 2011). Consequently, the rhizosphere microbiome may directly impact belowground competition by altering a plant’s extended niche (*i.e.,* microbes may mediate resource partitioning between plants; *reviewed in* Reynolds et al. 2002 and Bever et al. 2010) and/or indirectly by modulating root phenotypes. Root traits, however, can also influence the rhizosphere microbiome indirectly through their effects on the immediate soil environment or directly through carbon turnover of root biomass (Stres et al. 2008; Bach et al. 2010; Brockett et al 2012; Peralta et al. 2013; Wang et al. 2013; Spohn et al. 2014; Van Horn et al. 2014; Yan et al. 2015; Erktan et al. 2018). Therefore, root traits and the rhizosphere microbiome may impose additive and/or synergistic effects on plant fitness and function. As a result, this may have important consequences on how plants compete for resources belowground and may potentially alter phenotypic selection on plant traits, linking ecology and evolution. However, research examining if and how root phenotypes and the rhizosphere microbiome can potentially influence each other is limited. Furthermore, research taking into account both root traits and variation in the microbial community structure and composition of the rhizosphere as important predictors of plant fitness in the context of competitive environment remains unexplored. Here, as a first step, we addressed the broad question, Does rhizosphere bacterial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment?

Here, we extended our previous analysis of belowground competition (Colom and Baucom in Prep) to that of the rhizosphere microbiome. We used rhizosphere soil samples taken from our focal plant species, *Ipomoea purpurea,* grown in the presence and absence of competition from its sister species, *I. hederacea,* and asked two main questions: (1) Does the rhizosphere microbiome vary with phenotypic variation in root traits? (2) Does plant fitness vary as a function of root trait and rhizosphere bacterial community structure and/or composition, according to competitive environment (presence vs absence of belowground competition)? Addressing the first question would provide initial evidence that root phenotypes and the rhizosphere microbiome may influence each other, whereas addressing the second question would provide preliminary evidence for the potential for root phenotypes and/or the rhizosphere microbiome to influence plant fitness in context of belowground competition. Together, answering these main questions would provide evidence for the potential that the structure of plant roots and their rhizosphere microbiome may feedback into competitive belowground dynamics.

## Materials and methods

*Field experiment, rhizosphere soil collection--*We subsampled rhizosphere soil from individuals of *I. purpurea* and *I. hederacea* planted in the presence and absence of competition, with *I. purpurea* as the focal species in this experiment. For the competition treatment, we planted ten maternal lines of *I. purpurea* with six maternal lines of *I. hederacea*, for each possible maternal line by maternal line combination between species, which led to 60 unique competition pairings. We planted seeds 8 cm apart with 1 m2 between experimental units. For the alone treatment, we planted a single replicate seed of the ten maternal lines of *I. purpurea* 1 m2 apart. Each experimental unit was replicated sixteen times to yield a total of 2,080 seeds. Seven weeks post planting, when plants began to show signs of reproductive maturity, we excavated a subset of individuals to quantify root system traits (Colom and Baucom, 2020). We sampled the rhizosphere soil from 173 plants; 27 plants grown alone and 146 plants grown in competition. We randomly selected between 2 and 4 biological replicates of each *I. purpurea* maternal line grown alone, and between 5-12 biological replicates of each *I. purpurea* maternal line grown in competition, with the exception of one maternal line that had only one biological replicate. To isolate the rhizosphere soil from plant roots, we gently shook the roots from the soil cores of excavated plants to remove loose soil, sampled a random lateral root with small pieces of soil (~25mg) attached to its immediate surface (~1mm) with a 15mL sterile plastic tube, separated it from the rest of the root system with a razor that was cleaned with 90% ethanol, stored the tube immediately on dry ice, and later transferred all tubes to a -80C freezer until further use.

*DNA extraction and processing--*We extracted DNA from approximately 0.25g of rhizosphere soil per plant per standard procedures of the DNeasy PowerSoil Kit (QIAGEN, Hilden Germany), and then randomized 1uL of the DNA samples across two 96 well plates. The bacterial V4 region of the 16S rRNA gene was amplified and barcoded at the University of Michigan Medical School, and pooled libraries were sequenced on Illumina MiSeq sequencer, using v2 chemistry 2 × 250 (500 cycles) paired-end reads. Sequence quality processing was performed with mothur v1.43.0 using the MiSeq standard operating protocol (accessed on 31 October 2019) for the generation of the operational taxonomic unit (OTU 97% sequence similarity). For sequence alignment and classification, we used the SILVA release taxonomy (v132, Quast et al 2013; accessed August 2019), and only bacterial sequences were retained.

## Statistical analysis

All analyses were carried out in the statistical programming language R (R Core, 2019).

*Calculation of rhizosphere microbiome community composition and structure--*We aggregated our total OTU’s (52,565) at the genus taxonomic level to reduce patchiness in our data with the ‘tax\_glom’ function of the ‘phyloseq’ package (McMurdie and Holmes, 2013) which produced a total of 1,097 OTUs. We examined the distribution of sequencing depths of all our samples and filtered out extreme outliers (< 20,000 read counts) for all subsequent analyses. Sequence counts were used to compute different metrics of community composition including evenness, richness, Simpson diversity and Inverse Simpson diversity. Evenness quantifies how evenly represented different Bacterial taxa are as a proportion ranging from 0 to 1, where a value of 0 indicates lack of evenness in the community and towards 1 indicates a more even community. Richness is the total number of unique Bacterial taxa. Simpson diversity is a measure of community diversity that accounts for both species richness and their relative abundance. A Simpson Diversity measure of 0 indicates no diversity and increasing values indicates higher diversity within a given community. We also estimated the ‘effective’ species diversity (Inverse Simpson Diversity), a measure based on the Simpson Diversity at an order of 2 because it quantifies the effective number of different Bacterial taxa, wherein the weighted arithmetic mean is used to quantify average proportional abundance of types in the community. In practice, the Simpson Diversity Index can be used to measure the probability that two samples taken at random from the dataset represent the same taxon, whereas the Inverse Simpson Index can inform us the number of unique species weighted by their relative abundance. To estimate these metrics of ɑ-diversity, we used the function ‘estimate\_richness’ from the phyloseq package (McMurdie and Holmes, 2013), and specified the ‘measures’ argument for the corresponding metrics above. For each metric of ɑ-diversity we rarified to the number of sequences in the smallest sample. Then we normalized our sequences based on OTU read count data scaled to the smallest library size (Denef et al. 2017) and used the scaled data to compute community composition with the Bray–Curtis dissimilarity inter-community metric with phyloseq’s ‘ordinate’ function (McMurdie and Holmes, 2013).

*Characterizing sources of variation in bacterial community composition and structure—*We first examined how metrics of species structure and composition varied as a function of block, treatment and maternal line from rhizosphere microbiome collected from our focal species, *I. purpurea*. Preliminary histogram plots of bacterial species richness, evenness, Simpson Diversity, and Inverse Simpson Diversity showed normal distributions, hence, we elected to perform linear mixed model ANOVAs to test for these effects on our metrics of ɑ-diversity. We performed separate ANOVAs with the ‘lmer’ function from the lmerTest package (Kuznetsova et al. 2017), where we treated each ɑ-diversity metric as a response variable and included treatment and block as fixed effects and maternal line as a random effect. We excluded the interaction term between treatment and block because preliminary analysis did not show that these explained a significant portion of variation, nor did it improve the Akaike Information Criterion (AIC) of the model. Further, because none of our linear mixed models uncovered a significant maternal line effect on ɑ-diversity, we excluded this term in our final model and report the results of the two-way ANOVA, ɑ-diversity ~ Treatment + Block.

We performed a permutational ANOVA (PERMANOVA) to examine effects of block, treatment and maternal line on community composition using the ‘adonis’ function of the ‘vegan’ package (Oksanen *et al.* 2019) with default parameters and used 999 × permutations to access the significance of these variables for *I. purpurea* only. For this test, we treated community composition as our response variable and treatment, block and maternal line as fixed effects. Because preliminary analysis showed that maternal line did not explain a significant amount of variation in community composition we excluded this term from our final model and report the results of the model, β -diversity ~ Treatment + Block.

*Does bacterial community composition and structure vary with root traits?* To examine if and how different metrics of the rhizosphere microbiome community composition and structure are associated with phenotypic variation in root traits, we performed separate linear regressions for root architecture, size, topology and morphology, onto each metric of ɑ-diversity. We elected to focus on root architecture, size, topology and morphology because these traits can have direct impact on soil structure and plant resource uptake (Fitter 1987; Lynch 1995). To obtain our root traits, we applied multivariate statistics that transformed 33 root traits previously quantified from our experimental plants (Colom and Baucom, In Prep), into four modular traits. Specifically, we applied a Box-Cox transformation to all 33 root traits to normalize their distributions and standardized them by subtracting the mean and dividing by their standard deviations. Then we applied a PCA to their correlation matrix and elected to use the first four principal components (PCs) as our four modular traits because they each captured at least 10% of the total phenotypic variation each. We found that the first four PCs served as four important indicators of the root system: topology (PC1), or traits that describe the overall shape of the root system, architecture (PC2), or traits that capture the spatial arrangement of the root system (*e.g.,* different root tissue angle measurements, horizontal/vertical root distribution), size (PC3) (*e.g.,* root area) and morphology (PC4), or traits related to the individual characteristics of the root system (*e.g.,* root diameter estimates, basal root number and adventitious root number).

Briefly, a greater value of root topology (PC1), corresponds to a root system that exhibits a larger root width with a concomitant increase in soil depth. A greater value of root architecture (PC2) corresponds to a root system that grows more narrowly near the soil surface with an increase in the maximum root tissue angle. A greater value of root size (PC3) describes a root system that has a larger root surface area, and greater values of root morphology (PC4) correspond to a root system that has multiple lateral roots and smaller lateral root diameter and exhibits a greater range in the rooting angles relative to the soil surface and to the tap root. More details about how specific root traits contributed to each PC can be found in Chapter 3, *Describing the root system as modular root traits*).

To analyze the relationship between measures of bacterial community structure and phenotypic variation of root traits we performed separate linear regression analyses of root topology, architecture, size and morphology. We used our estimates of ɑ-diversity (*i.e.,* species richness, evenness, Simpson index, and Inverse Simpson index) as our predictor variables, and included treatment and block as additional predictors in all models. If we uncovered a significant linear relationship between a given root trait on ɑ-diversity, we also performed an ANCOVA using the ‘Anova’ function from the ‘car’ package (Fox and Weisberg, 2019) that included the interaction term of treatment by root trait. A significant root trait by treatment interaction would provide evidence that the competitive environment alters the relationship between a given root trait and measure of ɑ-diversity. We used *F-statistics* and Type III Sums of Squares to determine the statistical significance of fixed effects in the ANCOVAs.

Because root traits can significantly alter their immediate soil environment, we reasoned that greater phenotypic differentiation in root traits between plants could potentially promote greater differences in their corresponding rhizosphere communities. Accordingly, we evaluated whether greater phenotypic distance for a given root trait between individuals, was linearly linked to greater dissimilarity in their rhizosphere community composition. We used a Mantel test to evaluate evidence of a linear relationship between root phenotypes and community composition. For this analysis we calculated the Euclidean distance of root topology, architecture, size and morphology-- *i.e.,* ‘phenotypic distances’--between all plant samples (*i.e.,* across treatment and species), and then regressed each phenotypic distance onto the untransformed Bray-Curtis dissimilarity matrix with the ‘Mantel’ function from the vegan package (Oksanen *et al.* 2019) with the Spearman correlation method and 999 permutations. Because our PERMANOVA above did not uncover significant treatment effects on community composition (Table 2), we ran this test across treatment within *I. purpurea* (Table 4).

*Testing the effects of root traits and bacterial diversity measures on plant fitness*– We performed an ANCOVA to evaluate whether root traits and/or measures of bacterial diversity have direct effects on the fitness of *I. purpurea* according to treatment. A model that includes all root traits and metrics of ɑ-diversity controls for their correlations and provides us with an estimate of their directlinear effect on plant fitness, respectively. To estimate relative fitness, we used values of observed seed number collected from *I. purpurea* plants that were maintained until senescence (Colom and Baucom, In Prep), and divided this by the mean seed number by treatment. Then we averaged the relative finesses by treatment, block and maternal line. Before analysis, we scaled our measures of ɑ-diversity to a mean of zero and standard deviation of one. We fit a linear model that included treatment, block, root traits and standardized measures of bacterial species evenness, richness and Inverse Simpson Diversity and each of their two-way interactions with treatment and block as explanatory fixed effects (Relative fitness ~ Treatment + Block + Root topology + Root architecture + Root morphology + Sp. Richness + Sp. Inverse Simpson Diversity + Treatment × Block + Root topology × Treatment + Root architecture × Treatment + Root morphology × Treatment + Sp. Richness × Treatment + Sp. Inverse Simpson Diversity × Treatment + Root topology × Block + Root architecture × Block + Root morphology × Block + Sp. Richness × Block + Sp. Inverse Simpson Diversity × Block; Supplementary Information Table S3-1 for full model details). We did not include three-way interactions between treatment, block and root traits or between treatment, block and root traits and ɑ-diversity due to our limited sample size within block and treatment. Further, we excluded Simpson Diversity from this analysis as a predictor variable because it is strongly correlated to Inverse Simpson Diversity (*r* = 0.92, *p-value* < 0.001). We simplified our full model by doing a backwards model selection approach using the ‘stepAIC’ function from the MASS package (Venables and Ripley 2002) and retained the model with the lowest AIC (Relative fitness ~ Treatment + Block + Root topology + Root architecture + Root morphology + Sp. Richness + Sp. Inverse Simpson Diversity + Treatment × Block + Root topology × Treatment + Root architecture × Treatment + Root morphology × Treatment + Sp. Richness × Treatment + Sp. Inverse Simpson Diversity × Treatment + Root topology × Block + Root architecture × Block + Root morphology × Block + Sp. Richness × Block + Sp. Inverse Simpson Diversity × Block; Table S3-1). We used *F-tests* with Type III Sums of Squares to evaluate the significance of interaction terms using the ‘Anova’ function from the ‘Car’ package (Fox and Weisburg, 2019). A significant root trait by treatment or ɑ-diversity by treatment term would provide evidence that belowground competition alters the direct effects of root trait or ɑ-diversity on plant fitness, respectively. Likewise, a significant root trait by treatment or ɑ-diversity by treatment term would provide evidence that the competitive environment influences the direct effects of root trait or ɑ-diversity on plant fitness, respectively.

Since our ANCOVA showed evidence of a significant treatment by richness interaction effect on relative fitness (see *Evidence of linear relationships between root traits and bacterial diversity* below), we performed a linear regression of relative fitness as a function of nontransformed richness values for each treatment separately. We used *t-tests* to assess the significance of the linear relationship.

*Testing the effects of root traits and bacterial community composition on plant fitness–To* evaluate whether relative fitness of *I. purpurea* varies with itsrhizosphere bacterial community composition) and/or root traits,according to treatment, we performed a series of Mantel partial regressions. For each root trait we correlated the Bray-Curtis Dissimilarity matrix as a predictor variable and a vector of the Euclidean distances of a given root trait as a covariate (*i.e.,* ‘phenotypic distances’) onto a vector of the Euclidean distances of relative fitness of *I. purpurea*, for each treatment, separately. As above, analyzing community composition and root traits in the same model allow us to control for correlations between root traits and community composition and estimate their direct effects on plant fitness.

## Results

*Main effects of bacterial community composition and structure*--ANOVAs demonstrated that block was the biggest source of variation in ɑ-diversity metrics when examined across treatments (Richness: *F-value*3,95 = 5.29, *P*= 0.002; Inverse Simpson: *F-value*3,167 = 3.90, *P*= 0.01; Simpson: *F-value*3,95 = 2.76, *P*= 0.046; Evenness: *F-value*3,95 = 5.15, *P* 0.002; Table 1). Likewise, PERMANOVAs showed that block explained the biggest proportion of variation in species composition (*F-value*3,95 = 3.48, *P*= 0.001; Table 2). Competition treatment did not explain a significant proportion of the variation in ɑ-diversity metrics (Table 1) or species composition (Table 2). Together, these results show that the immediate soil environment is the main driver underlying the community composition and structure of the rhizosphere microbiome of *I. purpurea*.

*Evidence of linear relationships between root traits and bacterial diversity*—We uncovered a significant negative linear relationship between root architecture and bacterial species richness (*R*2 = 0.26, 𝛣 = -5.73 ± 2.22, *P*= 0.01; Table 3, Figure 1A) and a positive relationship with species evenness (*R*2 = 0.20, 𝛣 = 7.25 e-05 ± 3.19 e-05, *P*= 0.01; Table 3, Figure 1B). We also uncovered a significant positive relationship between root morphology and Simpson diversity (*R*2 = 0.23, 𝛣 = 2.48 e-03 ± 9.04 e-04, *P*= 0.001), however, visual inspection revealed an outlier (Simpson diversity = 0.94) was driving the linear trend between these two variables, and after removing the point the relationship was no longer significant (*R*2 = 0.10, 𝛣 = 0.001 ± 0.001, *P*0.36; Table 3). These results provide evidence that an increase in traits associated with root architecture (*e.g*., the maximum root tissue angle, basal root angle, root system width and root system length) is linked to a reduction in bacterial richness and simultaneous increase in species evenness. Interestingly, we uncovered no evidence that the presence of a competitor changed the direction of the relationship between root architecture and species richness.

We conducted Mantel tests to examine the potential for a linear relationship between Bray-Curtis Dissimilarity matrix, *i.e.,* community composition, and phenotypic distance in root topology, architecture, size and morphology. We did not find evidence of significant correlations between phenotypic distances of these root traits and community composition. However, we found a weak and marginally significant correlation between root architecture and community composition (*r2* = 0.07, *P*= 0.07; Table 4). These results indicate that while differences in root topology, size and morphology are not linearly associated with greater differences in the bacterial community composition, root architecture may be.

*Bacterial community structure effects plant fitness according to treatment--*We found evidence for a treatment by bacterial species richness interaction effect (*F-value1,57* = 5.03, *P*= 0.03; Table 5) on fitness, indicating that the bacterial community composition may play an important role in the outcome of competition in *I. purpurea*. Moreover, compared to the alone treatment, an increase in standardized bacterial species richness was linked to a decrease in plant fitness of -0.57 (*P*= 0.03), suggesting that more rich rhizosphere bacterial communities is associated to poorer plant performance in competitive belowground environments. Furthermore, we found evidence of an interaction between block and root topology (*F-value3,57* = 5.82, *P*= 0.002; Table 5) and root morphology (*F-value3,57* = 5.74, *P*= 0.002; Table 5), indicating that that the direct effects of root topology and morphology on plant fitness depend on environmental context. We likewise found a significant interaction between block and bacterial species richness (*F-value3,57* = 3.41, *P*= 0.02; Table 5), inverse Simpson (*F-value3,57* = 3.22, *P*= 0.03; Table 5), and evenness (*F-value3,57* = 3.50, *P*= 0.02; Table 5) indicating that the direct effects the rhizosphere community structure on plant fitness depend on environmental context.

We found no evidence of direct effects of root traits and measures of bacterial community composition, or β-Diversity, on relative fitness between treatments (results not shown).

## Discussion

The main goal of our work was to evaluate the potential for a relationship between modular root traits and the rhizosphere microbiome and to determine the relative impact of the rhizosphere bacterial community on plant fitness in context of belowground competition. Our findings reveal that multiple metrics of ɑ-diversity (bacterial richness, evenness and Inverse Simpson diversity) were linearly associated with different root traits, and that bacterial species richness may play an important role in belowground competition, as indicated by evidence for a significant two-way interaction effect between bacterial richness and competitive treatment on the relative fitness of *I. purpurea*. We also found a significant influence of block on the rhizosphere microbiome community composition and structure, but no evidence for an effect of competition, indicating that the community structure and composition of the rhizosphere microbiome in this species is influenced largely by the environment. Below we expand on the interpretation of our main findings and discuss their eco-evolutionary implications and directions for future research

***Associations between root traits and the rhizosphere microbiome***

The belowground root system of plants can play a major role in altering the physical and chemical profile of its surrounding soil environment (Orwin et al. 2010; Bodner et al. 2014) and therefore may serve as a passive filter of the bacterial community assemblage in the rhizosphere. For instance, lateral root type, seminal or nodal roots, has been found to influence the composition of rhizosphere bacterial communities in *Brachypodium* (Kawasaki et al., 2016). In turn, microbes residing in the rhizosphere can alter phenotypic plasticity of root traits by producing growth stimulating molecules and/or altering the chemical profile of the soil environment (*discussed in* Friesen et al. 2011). As such, we reasoned that root traits and the rhizosphere microbiome community are likely to influence each other, which may potentially impact downstream effects on plant function and fitness. In line with this broad expectation, we found a significant positive linear relationship between root architecture – a modular trait that captures the spatial arrangement of the root system – and bacterial evenness, and likewise a negative linear relationship between root architecture and bacterial richness. These results suggest that narrower, but deeper-growing root systems (*i.e.,* increased values of ‘root architecture’), are linked to a decrease in the presence of rare bacterial taxa (and vice versa). This would explain the simultaneous increase in bacterial evenness and decrease in bacterial richness with an increase in a more narrow/deep root system. Consistent with these results, we also found evidence of linear relationships between community composition and root traits, with a marginally significant positive correlation between community composition and root architecture, suggesting that specific root architectures in *I. purpurea* may play a role in differentiating the rhizosphere community between plants.

While we have identified these relationships between root architecture and bacterial richness and evenness, we have yet to test their mechanism. One plausible explanation for these findings is that root architecture influences its rhizosphere microbiome indirectly by altering soil moisture and/or access to nutrients, since root architecture can impact mineral aggregation and water flow in the soil (*reviewed in* Ghestem et al. 2011). Regardless of mechanism, our result that rhizosphere microbiome diversity varies with root architecture is in line with research from other plants, where research has uncovered associations between root system architecture traits and variation in rhizosphere bacterial communities (Szoboszlay et al. 2015; *discussed in* Saleem et al. 2018). For example, one study that compared the root system architecture and rhizosphere bacterial community of *Balsas teosinte* (progenitor of maize, *Zea mays* subsp. *Parviglumis*) and two domesticated corn cultivars, showed concurrent differences in rooting length and rhizosphere bacterial richness, composition and structure (Szoboszlay et al. 2015). In addition to bacterial community associations with architecture traits, we also found a significant positive relationship between Inverse Simpson Diversity and root morphology, indicating that root systems with an increase in lateral root number and decrease in overall root diameter (*i.e.,* thinner roots), support an increase in bacterial richness and relative abundance in the rhizosphere (and vice versa). This result may possibly reflect an increase in bacterial diversity through an increase in the available source of organic carbon in the soil from root litter (*discussed in* Reeder et al. 2001; Wardle et al. 2004; Bardgett et al. 2014), since thinner roots tend to have higher turn-over rates.

Consistent with this hypothesis, multiple studies have shown that root derived sources of carbon can alter soil bacterial community composition and structure (*discussed in* Reeder et al. 2001; Allison et al. 2006; Steenwerth et al. 2007) and some studies have reported positive associations between the abundance of particular bacteria (*e.g.,* Bacteroidetes) to thin root phenotypes in wild accessions of bean (Brown et al. 2012; Filippo et al. 2010; Pérez Jaramillo et al. 2017). These associations between root traits and rhizosphere bacterial communities, however, could also be due (at least partially) to rhizosphere linked microbes eliciting phenotypic plasticity of root architecture and/or morphology. For instance, many microbial taxa have been shown to influence root system architecture and morphological traits by synthesizing molecules that modulate the auxin pathway, *e.g.,* the production of phytohormones enhancing lateral root branching by plant growth promoting rhizobacteria (*reviewed in* Ortíz-Castro et al. 2009, Vacheron et al. 2013 and Sukumar et al. 2013; Bailly et al. 2014). Further, these patterns are also likely driven to some extent by microenvironmental changes in soil conditions because it can trigger both phenotypic plasticity of root traits and alter microbial niches and influence microbial communities (Bonser et al. 1996; Hodge 2004; Gruber et al. 2013; Tian et al. 2014; Yu et al. 2014; Bach et al. 2010; Brockett et al. 2012; Zhalnina et al. 2015).

***Evidence for the potential of the rhizosphere microbiome to impact belowground competition***

Given that the primary role of root traits is to acquire nutrients and water from the soil environment, and that the rhizosphere microbiome can strongly influence the bioavailability of key resources and thus plant fitness, we hypothesized that root traits and/or bacterial diversity may influence how plants respond to the stress of competition. We found that bacterial species richness had a significant positive linear relationship with plant fitness in the presence of competition, but no relationship in the absence of competition, suggesting that an increase rhizosphere species richness improves *I. purpurea*’s fitness when in competition. Thus, while belowground competition negatively impacts *I. purpurea*’sfitness (Colom and Baucom 2020; results in Chapter 3), our findings perhaps indicate that bacterial richness can ameliorate the negative effects of plant competition. However, we also identified a relationship between rhizosphere diversity metrics and root phenotypes, meaning that the effect on fitness we have identified here could simply be due to the effect of root phenotypic traits on plant fitness. To delineate the importance of the root phenotype versus metrics of rhizosphere diversity on plant fitness, we would need to assess the fitness of plants with different root architectures while experimentally altering **both** the bacterial diversity in the soil and the competitive environment.

If bacterial richness does indeed influence plant fitness while in competition, one possible explanation is that the bacterial community may lead to an increase in the bioavailability of essential nutrients *via* an increase in bacterial functional richness. For instance, Singh and others (2015) performed a controlled greenhouse experiment where they grew *Ocimum sanctum* (basil) plants in potting soil that was inoculated with different levels of bacterial species diversity and richness and found that richness was an important predictor of increased plant biomass. Further, they found that the functional group richness of bacterial species was positively associated with plant biomass, suggesting the potential for increase in rhizosphere bacterial richness to promote plant growth via an increase in bacterial function. While research examining the influence of both root traits and the rhizosphere microbiome on plant fitness remains scarce, multiple studies have shown that altering the soil microbial community can alter plant performance according to competitive environment (Callaway et al. 2004; Lankau 2010; *discussed in* Bever et al. 2010; Larios et al. 2015), highlighting the importance of plant-microbial interactions to influence belowground competition.

**Conclusion**

Understanding how root traits and their associated microbial communities may influence belowground competition and feedback into plant ecology and evolution is an elusive challenge in evolutionary ecology. As a first step, we demonstrated here that root traits and the rhizosphere microbiome are related, providing initial evidence that root phenotypes and the rhizosphere bacterial community may influence each other. We also found evidence that an increase in bacterial species richness can have a positive impact on plant fitness when plants experience belowground competition, suggesting that the rhizosphere microbiome can potentially mitigate the harmful effects of belowground competition. Therefore, our work provides preliminary evidence that interactions between root traits and the rhizosphere bacterial community may perhaps feedback into belowground competition thus potentially alterplant ecology and evolution. We emphasize, however, that the underlying mechanisms producing many of the patterns we uncovered are yet to be determined because we did not manipulate the rhizosphere microbial community and/or root traits. Furthermore, we also found that unmeasured aspects of the environment (*i.e.,* block effects) significantly influence the rhizosphere microbiome. Therefore, future work that manipulates the rhizosphere microbiome, soil conditions and/or root traits will be essential for disentangling different ecological factors and drawing causal inferences.

While our work serves as a first step towards understanding the potential for plants and their rhizosphere microbiome to feedback into dynamics of belowground competition, we are considerably limited in that fungal organisms were not evaluated as part of the rhizosphere microbiome here. As a result, we excluded many functionally relevant species that contribute to plant resource use and fitness (Jonsson et al. 2001; Bassirad 2005; van der Heijden et al. 2006 and 2008; Jacoby et al. 2017). Thus, consideration of both bacterial and fungal species in future work will be required in order to develop a more realistic view on how root traits and the rhizosphere microbiome may potentially feed back into processes that shape plant evolution and diversity.

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**Author contributions**

SMC and RMB developed and designed the research. SMC collected, processed, and extracted DNA from the soil samples, and collected/quantified plant root/fitness traits. All authors contributed to data analysis and to write and revise the text.

**Data availability statement:**The R code is available at GitHub at <https://github.com/SaraMColom/Microbiome_2018> and the data will be uploaded to the Dryad Digital Repository.

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**Tables**

|  |  |  |
| --- | --- | --- |
| ɑ-Diversity | Treatment *df* = 1 | Block *df* = 3 |
| Richness | 1.04 (0.31) | **5.29(0.002)** |
| Inverse Simpson | 0.03 (0.87) | **3.90 (0.01)** |
| Simpson | 0.28 (0.60) | **2.76 (0.046)** |
| Evenness | 1.03 (0.31) | **5.15 (0.002)** |

Table 1 Results from separate ANOVAs to test for Treatment effects on different alpha diversity metrics (ɑ-Diversity Metric) of the rhizosphere microbiome of *I. purpurea* (Num. *I. purpurea* in competition = 73; Num. *I. purpurea* alone = 27). *F-values* are reported with their corresponding *p-values* in parentheses. Each modelǂ evaluated metrics of ɑ-Diversity as response variables, and Treatment and Block as fixed effects. Values in bold indicate a significant *p-value* < 0.05.

ǂ*ɑ-diversity* ~ Treatment + Block

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Effect | DF | SS | MeanSS | *F-value* | *R*2 | *p-value* |
| Treatment | 1 | 0.03 | 0.03 | 0.85 | 0.01 | 0.55 |
| Block | 3 | 0.32 | 0.11 | **3.48** | **0.10** | **0.001** |

Table 2 Results of PERMANOVA of Bray-Curtis distances (community composition) to test for Treatment effects on community composition of the rhizosphere microbiome of *I. purpurea* (Num. *I. purpurea* in competition = 73; Num. *I. purpurea* alone = 27). The modelǂ included community composition as a response variable, with Treatment and Block as fixed effects.

ǂ*Community composition* ~ Treatment + Block

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Linear Association Between Rhizosphere Microbiome ɑ-Diversity and Root Traits | | | | |
| ɑ-Diversity | Root topology  (PC1) | Root architecture (PC2) | Root size  (PC3) | Root morphology (PC4) |
| Inverse Simpson | 1.02  ± 1.43 | -0.56  ± 0.73 | 0.53  ± 0.89 | 1.90^  ± 1.09 |
| Simpson | 1.45 e-03  ± 1.26 e-03 | -9.07 e-04  ± 6.32 e-04 | 9.58 e-04 ± 7.58 e-04 | **2.48 e-03\*\***  **± 9.04 e-04** |
| Richness | 3.38  ± 4.65 | **-5.73\***  **± 2.22** | 2.00  ± 2.81 | 3.50  ± 3.52 |
| Evenness | -6.20e-06  ± -6.62 e-05 | **7.25 e-05**  **± 3.19 e-05** | 2.84 e-06  ± 4.00 e-05 | 2.62 e-05  ± 5.02 e-05 |

Table 3 Results of separate linear regression between different metrics (ɑ-Diversity Metric) of the rhizosphere microbiome (Inverse Simpson, Simpson, Richness and Evenness) and four root traits (Root topology, Root architecture, Root size and Root morphology) examined in *I. purpurea*. ɑ-Diversity metrics were treated as response variables for each root trait, and Block and Treatment were included in the final modelǂ as fixed main effects.Linear regression coefficient slopes (𝛣) are reported with ± 1 standard error.

*p-value* < 0.05 \*; *p-value* <0.01 \*\*; *p-value* <0.001\*\*\*; *p-value* =0.09 ^

ǂModel: ɑ-Diversity ~ Root trait + Block +Treatment

|  |  |  |
| --- | --- | --- |
| Table 4 Mantel test Bray-Curtis and root phenotypes within *I. purpurea* | | |
| Root trait | *r2* | *p-value* |
| Root topology | -0.04 | 0.76 |
| Root architecture | 0.07 | 0.07^ |
| Root size | 0.07 | 0.13 |
| Root morphology | -0.04 | 0.76 |

*p-value* =0.07 ^

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Fixed effect** | **DF** | **SS** | **F-value** | **P-value** |
| Treatment | 1 | 0.02 | 0.27 | 0.61 |
| Block | 3 | 0.71 | 4.08 | **0.01** |
| Treatment × Block | 3 | 0.20 | 1.17 | 0.33 |
| Root topology × Treatment | 1 | 0.13 | 2.28 | 0.14 |
| Root size × Treatment | 1 | 0.06 | 0.97 | 0.33 |
| Richness × Treatment | 1 | 0.29 | 5.03 | **0.03** |
| Evenness × Treatment | 1 | 0.08 | 1.34 | 0.25 |
| Root topology × Block | 3 | 0.84 | 4.85 | **0.004** |
| Root architecture × Block | 3 | 0.26 | 1.48 | 0.23 |
| Root size × Block | 3 | 0.26 | 1.47 | 0.23 |
| Root morphology × Block | 3 | 1.15 | 6.60 | **<0.001** |
| Richness × Block | 3 | 0.60 | 3.42 | **0.02** |
| Inverse Simpson diversity × Block | 3 | 0.56 | 3.22 | **0.03** |
| Evenness × Block | 3 | 0.61 | 3.50 | **0.02** |

Table 5 Results of ANCOVA to test the effects of root traits (root topology, architecture, size and morphology, respectively), measures of alpha diversity of the rhizosphere microbial community, and their two-way interactions with Treatment and Block on relative fitness of *I. purpurea* (N = 100). Degrees of Freedom (DF), sum of squares (SS) and *F-values* and corresponding *p-value* in parentheses are reported for each fixed effect. For this analysis, we extrapolated observed values of root traits and alpha diversity metrics scaled to a mean of zero and standard deviation of one, onto relative fitness of *I. purpurea* averaged by maternal line and treatment. The final model included all the Fixed Effects listed in the table regressed onto relative fitness and *F-tests* with Type III Sums of Squares were used to estimate their statistical significance. *p-value* < 0.05 \*; *p-value* <0.01 \*\*; *p-value* <0.001\*\*\*