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Climate and edaphic controllers influence rhizosphere community assembly for a wild annual grass

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Abstract

The interface between roots and soil, known as the rhizosphere, is a dynamic habitat in the soil ecosystem. Unraveling the factors that control rhizosphere community assembly is a key starting point for understanding the diversity of plant-microbial interactions that occur in soil. The goals of this study were to determine how environmental factors shape rhizosphere microbial communities, such as local soil characteristics and the regional climate, and to determine the relative influence of the rhizosphere on microbial community assembly compared to the pressures imposed by the local and regional environment. We identified the bacteria present in the soil immediately adjacent to the roots of wild oat (*Avena spp*.) in three California grasslands using deep Illumina 16S sequencing. Rhizosphere communities were more similar to each other than to the surrounding soil communities from which they were derived, despite the fact that the grasslands studied were separated by hundreds of kilometers. The rhizosphere was the dominant factor structuring bacterial community composition (38% variance explained), and was comparable in magnitude to the combined local and regional effects (22% and 21%, respectively). Rhizosphere communities were most influenced by factors related to the regional climate (soil moisture and temperature), while background soil communities were more influenced by soil characteristics (pH, CEC, exchangeable cations, clay content). The *Avena* core microbiome was strongly phylogenetically clustered according to the metrics NRI and NTI, which indicates that selective processes likely shaped these communities. Furthermore, 17% of these taxa were not detectable in the background soil, even with a robust sequencing depth of approximately 70,000 sequences per sample. These results support the hypothesis that roots select less abundant or possibly rare populations in the soil microbial community, which appear to be lineages of bacteria that have made a physiological tradeoff for rhizosphere competence at the expense of their competitiveness in non-rhizosphere soil.

Key words: Avena; climate; community assembly; microbiome; plant-microbial interactions; rhizosphere; soil.

Introduction

Soil is a complex medium that harbors vast amounts of microbial life (Fulthorpe et al. 2008). The millimeters of soil that directly surround a root, referred to as the rhizosphere, form a microhabitat that plays a crucial role in all vegetated ecosystems. As a root grows through soil, it interacts with the indigenous soil organisms and develops a microbiome that can facilitate the acquisition of nutrients by the plant (Marschner et al. 1986), defend the plant from pathogens (Doornbos et al. 2012), alter decomposition rates of organic material (Cheng 2009), and change the fitness of the plant host (Panke-Buisse et al. 2015). Recent evidence suggests that the rhizosphere can influence the bacteria that colonize the root's surface and interior (Edwards et al. 2015). Therefore, the factors that affect the selection of the rhizosphere microbiome are important to plant fitness and may impact ecosystem function (Wagg et al. 2014).

Rhizosphere microbial communities occupy a niche that is governed in part by the plant root, the edaphic characteristics of the soil, the regional climatic conditions, and the interactions between these factors (Berg and Smalla 2009, Philippot et al. 2013). The processes that influence the acquisition of the rhizosphere microbiome are currently poorly understood (van der Heijden and Schlaeppi 2015). Roots are thought to influence microbial community assembly by producing an array of metabolites that vary with plant species and environmental conditions (Dennis et al. 2010), including secondary metabolites such as antimicrobial compounds (Bais et al. 2006). Roots also create a distinct soil microhabitat by altering the pH, porosity, and oxygen concentrations in the soil surrounding the root (Hinsinger et al. 2003, Blossfeld et al. 2013). The composition of the rhizosphere is specific to particular plants (Haichar et al. 2008), and the strength of the rhizosphere effect appears to differ between plants (Bulgarelli et al. 2013). In some cases, despite the selective influences discussed above, soil type can be the dominant factor structuring rhizosphere bacterial communities (e.g., Bulgarelli et al. 2012, Lundberg et al. 2012, Peiffer et al. 2013). Yet, in other systems, the plant can be the dominant factor structuring the rhizosphere bacterial communities (e.g., Germida et al. 1998, Wieland et al. 2001, Costa et al. 2006). Our knowledge of the rhizosphere is mostly derived from plants grown in managed environments (agricultural, greenhouse, or experimental field settings) with few studies conducted in uncultivated settings (Kowalchuk et al. 2002, Dean et al. 2015); the factors that structure rhizosphere communities in uncultivated settings are likely to differ from those in managed environments (Philippot et al. 2013). Furthermore, while recent rhizosphere studies identify a core microbiome, few studies have quantified the phylogenetic coherence of these communities (Shi et al. 2015), which has implications for the mechanisms underlying community assembly (Fine and Kembel 2011, Stegen et al. 2013).

Microbial community assembly is the study of the processes that shape microbial communities, and can be generally categorized as selection (biotic

or abiotic), drift, speciation, or dispersal (Vellend 2010, Stegen et al. 2013). Community assembly can be studied by observing communities over time, or by observing communities over a range of variables across space (Nemergut et al. 2013). The common annual grass, *Avena spp*. (wild oat), provides a unique opportunity for studying rhizosphere community assembly across a range of soil types and geographic locations. Grasslands are important terrestrial ecosystems, and account for approximately 14% of the world's land area, which is twice the land area cultivated for agriculture (Gong et al. 2013). *Avena* spp. populates Mediterranean grasslands worldwide (Clayton et al. 2006), and has been ubiquitous in California grasslands since the 1860s (Robbins 1940). As an annual, *Avena* establishes new rootstock each growing season that is colonized anew by the soil microbial community. *Avena* also reproduces clonally and thus has low levels of genetic variation, and *Avena barbata* in particular has extremely low levels of genetic variation in California (Latta 2009). The widespread distribution of *Avena* and low genetic diversity make it an excellent plant for assessing the abiotic factors that structure the rhizosphere microbiome in uncultivated field environments.

During the establishment of a rhizosphere community, the selective pressure exerted by a root competes with the pressures imposed by the local soil environment and the regional climate (Bulgarelli et al. 2013, Philippot et al. 2013). Our primary goals were (1) to determine which environmental factors shape rhizosphere microbial communities, such as local soil characteristics and the regional climate, and (2) to determine the relative strength of the rhizosphere effect on microbial community assembly compared to the pressures imposed by the local and regional environment. We hypothesized that soil characteristics (local or regional) would be the main factors influencing rhizosphere microbial community assembly for this wild annual grass. Alternatively, if instead the rhizosphere effect overwhelmed the influence of the local and regional soil environment, we hypothesized the core *Avena* microbiome would contain phylogenetically coherent lineages, which would indicate that selection is the dominant factor shaping these communities (Stegen et al. (2012). To test these hypotheses, we examined natural stands of a common annual grass, *Avena spp*., in three Mediterranean grasslands in California that encompass a range of soil and climatic characteristics (Fig. 1). The soils sampled were untilled and contained a densely rooted, mixed plant community, where the top 10 cm of soil can contain almost half of the total root biomass in the ecosystem (Jackson et al. 1996). *Avena* rhizosphere and surrounding soil communities (background soils) were characterized using deep Illumina 16S sequencing (ca 70 000 reads per sample). Sampling in-situ rhizosphere communities required developing a technique for extracting DNA from small volumes of soil (0.005 g). The background soils were characterized for a wide range of chemical and physical characteristics, as well as recent soil climatic characteristics that were measured over time (e.g., 8-month soil

temperature; soil moisture at time of harvest, week prior, and during the height of summer dryness). This sampling design allowed us to evaluate the influence of edaphic characteristics and climate on rhizosphere community assembly across a wide variety of soil types. The factors regulating the assembly of rhizosphere microbial communities are necessary to understand the development of rhizosphere microbiomes in terrestrial ecosystems, and ultimately to predict the response of these communities to environmental perturbations and climate change.

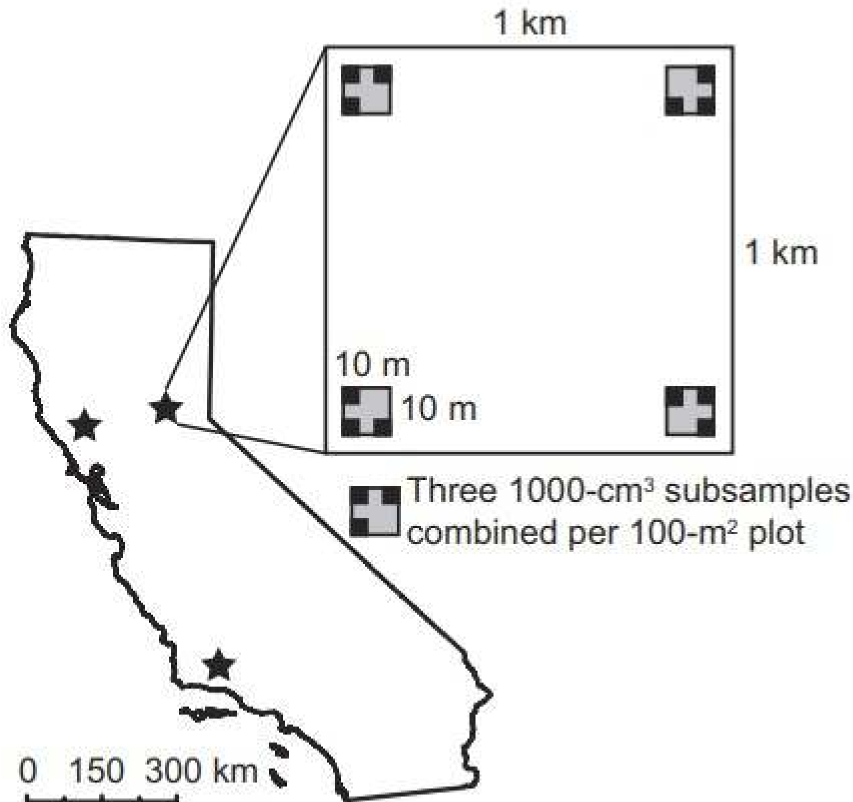


FIG. 1. Collection of experimental soils. Soil was collected from roots of wild oat (*Avena* spp.) and the surrounding background soil in three Mediterranean grasslands in California: Hopland (north coast), Sierra Foothills (north central), and Sedgwick (south coast). The plots were subsampled three times, and the soil was combined to create one representative sample for the plot. Each subsample consisted of a 1000-cm^3 volume of soil.

Methods

Experimental design

We investigated the controllers of in-situ rhizosphere microbial community composition associated with the common annual grass, *Avena* spp., at three California annual grasslands: Hopland Research and Extension Center

(Mendocino County, USA), Sierra Foothills Research and Extension Center (Yuba County, USA), and Sedgwick Reserve (Santa Barbara County, USA). *Avena* spp. are widespread at each site; a putative ecotype of *Avena barbata* currently dominates the Hopland and Sierra sites (Gardner and Latta 2006, Latta 2009), while *Avena fatua* dominates the southern California Sedgwick site.

At each grassland, four 100-m^2 plots were sampled at the corners of 1-km^2, for a total of 12 plots (Fig. 1). These soils encompassed a range of soil types, textures, and parent materials (Appendix S1: Table S1). Three 1000-cm^3 subsamples of soil were unearthed at the corners of the 100-m^2 plot (Appendix S1: Fig. S1A). Subsamples were characterized individually and later averaged to create a representative plot value. Harvests occurred after *Avena* inflorescences had emerged but prior to seed development. Soil was collected to measure water content 1 week prior to harvest, at the time of sampling, and during the dry summer after sampling (>3 months without rain). Soil harvests were staggered in spring 2010 due to the timing of grass flowering: late March (Sedgwick), mid-April (Sierra Foothills), and early May (Hopland).

Soil and plant characterization

For each 1,000-cm^3 subsample, two *Avena* plants with attached root systems were separated from the other plants (Appendix S1: Fig. S1B). Clumps of soil larger than 2 mm were removed from the roots. Roots were transported on dry ice and stored at −80°C. All remaining roots were removed from the soil. The subsample was homogenized and a subset of the soil (hereafter called "background" soil) was frozen on dry ice. The remaining soil was transported on blue ice, stored at 4°C, and processed within 2 d of harvest to measure pH, dissolved organic carbon (DOC), microbial biomass, and soil moisture, and soil C:N (see Appendix S1). Soil texture (sand, silt, clay), phosphorus (Bray), iron (DTPA), exchangeable cations (K, Na, Ca, Mg), and salinity (electrical conductivity, EC) were characterized at the UC Davis Analytical Laboratory. Soil temperature was measured every 2 h at 10 cm depth from January 2010 – August 2010 using iButton temperature data loggers (Maxim Integrated) (See Appendix S1).

Avena shoots and the remaining biomass per 100-cm^2 were collected to calculate *Avena* biomass and total aboveground biomass, respectively. Biomass was dried at 50°C for 3 d.

Rhizosphere and background soil collection

Two 3.2 cm root sections were washed per plant and combined for the three subsamples (Fig. 1), which yielded 0.005–0.02 g root-attached soil per site (six plants; 38.1 cm root total) (See Appendix S1). To minimize the variability in the roots sampled, we harvested roots that were light yellow and approximately 1 mm in diameter, and did not sample dark brown roots that were potentially decomposing or short crown roots that were potentially

younger than the other roots in the root system. Root debris down to approximately 1–2 mm in length was removed manually from supernatant using a flame-sterilized needle after each round of vortexing (see Appendix S1). To acquire a comparable amount of background soil, a total of 0.5 g soil was composited from the three subsamples and washed at the same soil:buffer ratio as the root samples, and then handled in the same manner as the rhizosphere samples to acquire 0.02 g soil.

Rhizosphere sampling evaluation

To assess if 0.005 g of rhizosphere soil was a sufficient sample size to generate a representative microbial community, we subsampled the roots in quadruplicate from eight 1,000-cm^3 volumes of soil collected at logarithmic distances from a starting point in Hopland, CA: 0 m (Hopland), 10 cm, 1 m, 10 m, 100 m, 1 km, 150 km (Sierra), 550 km (Sedgwick). The quadruplicates were extracted independently and prepared for sequencing in parallel with the other samples from this study.

DNA extraction and sequencing

DNA was extracted from 0.005 g rhizosphere or background soil using a modified phenol-chloroform method (Griffiths et al. 2000) (see Appendix S1 for modifications), which yielded 100–200 ng of DNA. The V4 region of 16S was amplified using 5 ng of DNA template (Caporaso et al. 2011) and sequenced in a single lane on an Illumina GAIIX sequencer (150 bp paired-end). Reverse reads were discarded due to short read lengths. Sequences were processed using QIIME (Caporaso et al. 2010), where chimeras, alignment failures, singletons, and sequences present in only one sample were removed from the dataset (see Appendix S1). Chloroplast and mitochondrial sequences were not removed from the dataset, and only represented a small fraction of the rarified data (0.065% and 0.048% total relative abundance, respectively), indicating minimal plant DNA contamination in these samples. Sequence data is available at NCBI BioProject accession PRJNA246258.

Data analysis

Soil characteristics

Soil edaphic characteristics were compared among sites using one-way ANOVA and *P*-values were adjusted to control the false-discovery rate using the Benjamini–Hochberg method using R. Non-normal data was transformed (see Appendix S1: Table S2 for transformations).

Beta-diversity

Sequences were rarefied to 69 884 sequences per sample (12,358 ± 1752 OTUs). For the rhizosphere sampling evaluation, sequences were rarefied to 16 178 sequences per sample (9840 ± 3887 OTUs), as some samples had fewer sequences in this experiment. Community distance matrices were calculated using weighted unifrac and ordinated using non-metric

multidimensional scaling (NMDS) in vegan (R: metaMDS) (Oksanen et al. 2013). Community data was clustered in one-dimension using hierarchical clustering (R: hclust).

Rhizosphere sampling evaluation

Replicate groups for the rhizosphere sample evaluation were analyzed for significant differences using a one-way PERMANOVA (R: adonis). If communities derived from 0.005 g soil were reproducible, we would expect samples to cluster by location in ordination space. We also tested for differences in beta-dispersion to determine if the variance within replicate groups differed among samples (R: betadisper).

Variance partitioning

Variance in the unifrac community distance matrix was partitioned across the factors Region (Sedgwick, Sierra, Hopland), Habitat type (Rhizosphere vs. Background soil), and Local Plot (100-m^2 site) using a two-way PERMANOVA (R: adonis). The data is nested, so we used an additional nested non-parametric MANOVA (R: nested.npmanova) to obtain the correct permutations and error terms (see Appendix S1).

Core microbiome

We assessed if the *Avena* rhizosphere had a core microbiome that was consistently detected across the grasslands studied, and if so, if these taxa were more phylogenetically related than expected by chance. Taxa that were enriched or depleted in the rhizosphere must be present in at least 50% of the rhizosphere or 50% of the background soil communities, respectively, and significantly differ in relative abundance by a paired t-test ($n \geq 6$, P-values Benjamini–Hochberg corrected). Net relatedness (NRI) and nearest taxon (NTI) indices were used to determine if the *Avena* core microbiome was significantly phylogenetically clustered (Webb et al. 2002). NRI and NTI are presented in units of standard deviation (10 000 randomizations; values >1.96 indicate significant phylogenetic clustering) (Vamosi et al. 2009).

Correlation analysis

We fit each environmental variable measured in this study as a vector in NMDS space to determine how well these variables correlated with the rhizosphere or background soil microbial communities (R: envfit, 10,000 randomizations) (see Appendix S1: Table S2 for full list of variables). The influence of *Avena* species on the rhizosphere communities was assessed separately using a constrained analysis of principle components (CAP), which controlled for the environmental variables (soil moisture, clay) that most strongly correlated with the rhizosphere microbial communities according to BIO-ENV (R: bioenv, capscale). To check if *Avena* species was confounded by the site effect, we compared data from a larger study collected at the same

time and sites as the current study, where *A. barbata* was sampled at Sedgwick (see Appendix S1: Fig. S2 for information).

Phylogenetic trees

Maximum likelihood (ML) trees were constructed from the Illumina sequences in FastTree (Price et al. 2010) using a constraint tree to initialize the topology (generalized time reversible model, gamma settings). To create the comprehensive ML tree for NRI and NTI, one full-length analog for each family was selected to build the constraint tree (483 family representatives), and the full tree was built from the short-read sequences. To create the core microbiome ML tree for data visualization, OTUs with full-length analogs were selected to build the constraint tree (1,031 OTU representatives), and the tree was again built from short-read sequences.

Results

Soil characteristics

The three grasslands studied differed in terms of climate, parent materials, and soil taxonomy (Appendix S1: Table S1), as well as edaphic characteristics (Appendix S1: Table S2). Sedgwick soils had significantly higher clay content, cation exchange capacity (CEC), and alkalinity (6.6–10 times lower H^+ concentrations). Sedgwick typically receives half to a third of the rain received at the other two grasslands and was the driest at the time of sampling. Sierra soils had a slightly but significantly higher C:N ratio and higher Ca content. Hopland soils encompassed multiple soil taxonomies, and had significantly lower CEC, Na, and Ca.

Rhizosphere sampling evaluation

We recovered 0.005–0.02 g of rhizosphere soil from wild *Avena* roots collected from the field. When extracting DNA from soil, most protocols typically use two orders of magnitude more material (0.25–0.5 g soil). Analysis of quadruplicate samples taken at 8 distances (0.1 m – 500 km) indicated that 0.005 g sufficiently captured the community composition, as shown by the replicate groups forming defined clusters in NMDS ordination space (Fig. 2). Overall, the differences among locations were significant (One-way PERMANOVA: $F_{7, 24} = 16$, $n = 4$, $P = 0.00010$). While there is some variability in the dispersion of the replicates (e.g., the 1 m replicate group), the replicate groups did not have significantly different variance in ordination space (Beta-dispersion: $F_{7, 24} = 2.0$, $n = 4$, $P = 0.091$; Tukey HSD: $n = 4$, $P > 0.10$).

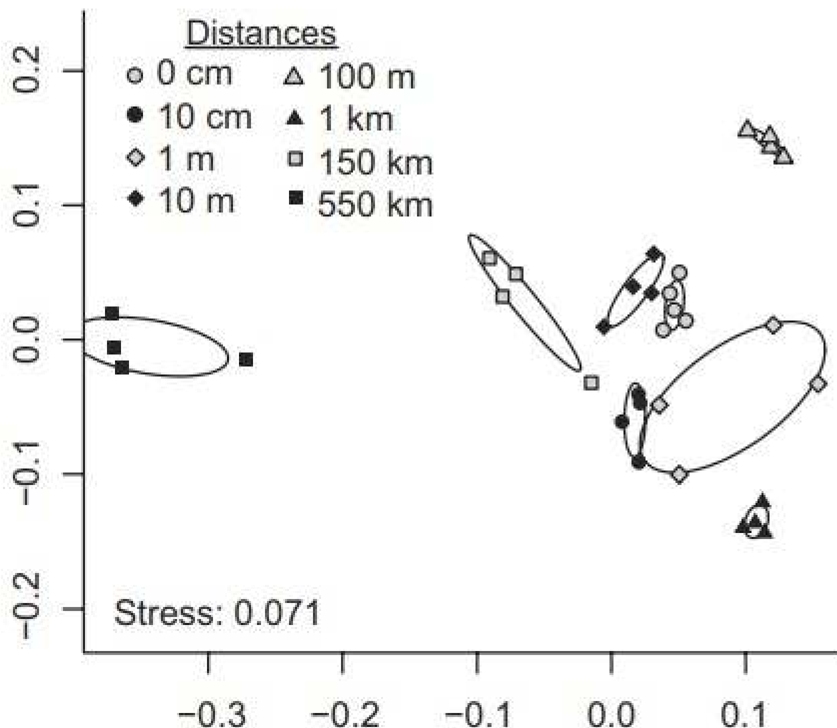


FIG. 2. Nonmetric multidimensional scaling ordination testing the reproducibility of 0.005 g rhizosphere soil samples. Samples were collected along a logarithmic transect (Hopland: 0 cm – 1 km; Sierra: 150 km; Sedgwick: 550 km). Ellipses indicate the 95% standard error from the mean centroid.

Soil microbial community controllers

In terms of community composition, the rhizosphere soils were more similar to each other than the background soils from which they were derived (Fig. 3A,B). The rhizosphere effect was comparable in magnitude to the combined local and regional effects (Fig. 3C), where the distinction between habitat types (rhizosphere versus background soil) explained 38% of the variance present in the community composition (Two-factor nested PERMANOVA: $F_{1,\,23}$ = 37, P = 0.00069), while region (grassland) and local soil conditions (sampling site) explained 21% and 22% of the variance, respectively (Nested non-parametric MANOVA: $F_{2,\,23}$ = 4.3, P = 0.00030; Two-factor nested PERMANOVA: $F_{9,\,23}$ = 2.4, P = 0.0018). The rhizosphere communities appeared to be more strongly influenced by regional effects and grouped by grassland, while the background soils were more similar to each other (Fig. 3A). The interaction between habitat type and region accounted for 9.3% of the variation, but was marginally significant (Two-factor nested PERMANOVA: $F_{2,\,23}$ = 4.5, P = 0.064).

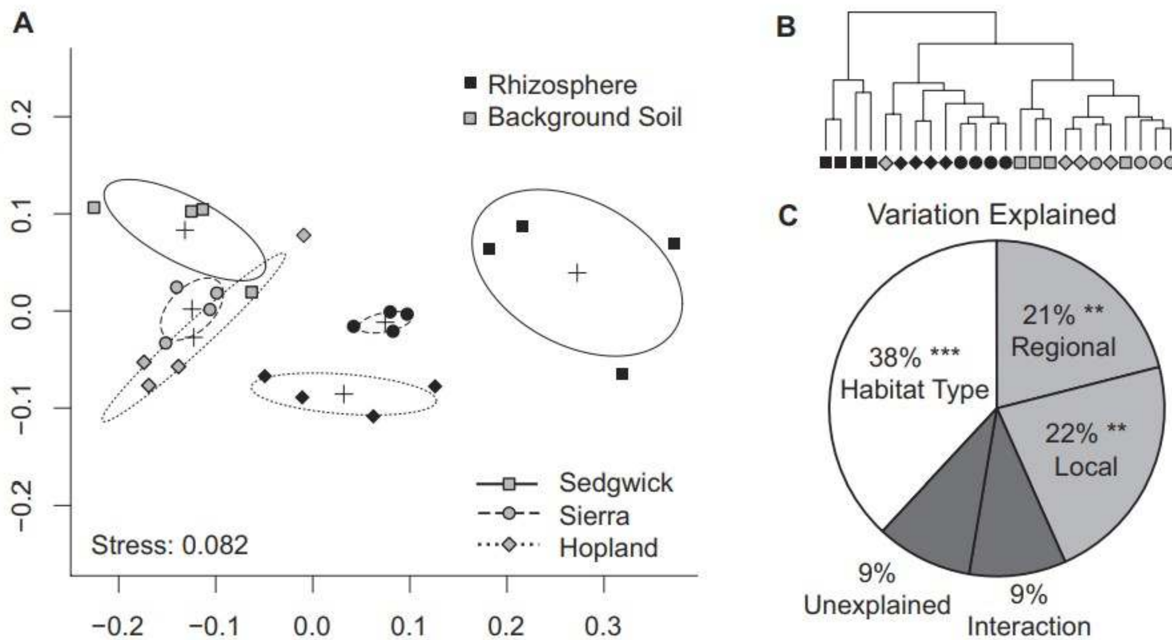
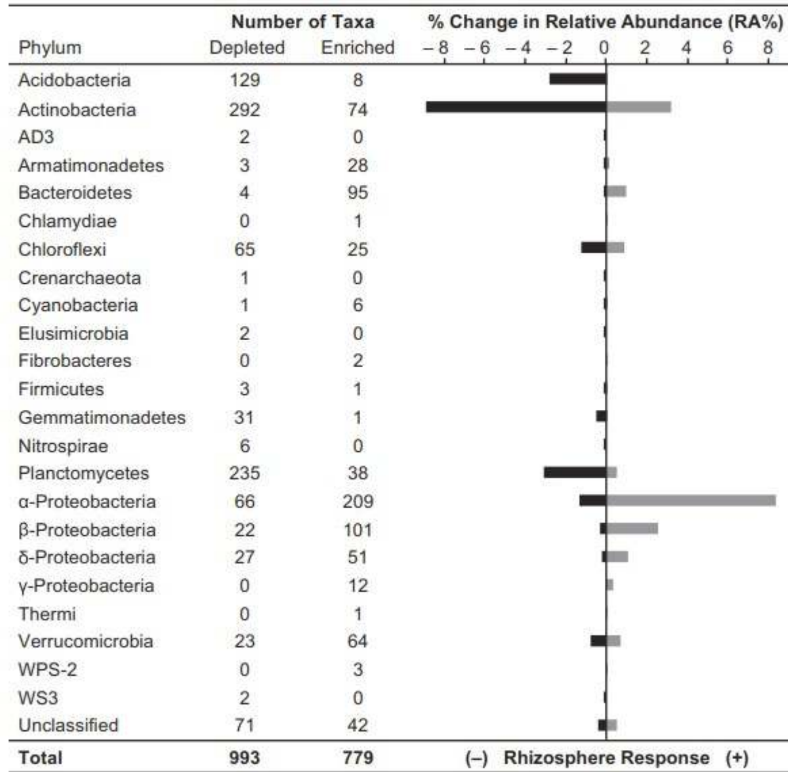


FIG. 3. (A) Non-metric multidimensional scaling ordination of the rhizosphere (black symbols) and background soil (grey symbols) communities at three California grasslands: Hopland (diamonds), Sierra (circles), Sedgwick (squares). Ellipses indicate 95% standard error from the mean centroid. (B) Hierarchical clustering diagram displaying the rhizosphere and background soil communities in one dimension. (C) Variance in the microbial community distance matrix explained by habitat type (Rhizosphere or Background soil), climatic region (Region), or the 1000-cm^3 volume of soil (Local). Stars indicate significance (***$P < 0.001$, **$P < 0.01$).

Core *Avena* rhizosphere microbiome

Members of the core community were present in at least half the rhizosphere samples and significantly enriched in the rhizosphere relative to the background soil. The core microbiome is presented in two ways: as the number of taxa significantly enriched or depleted in the rhizosphere, and as the average percent change in total relative abundance (Fig. 4, Appendix S1: Tables S3 and S4). Interestingly, even with a robust sequencing depth, 17% of the 779 taxa in the *Avena* core microbiome were not detectable in the background soil, while 7.6% of the 993 taxa depleted in the rhizosphere soil were only detectable in the background soil (Fig. 5 ring 2, Appendix S1: Table S4). These taxa had low abundances in the rarified dataset, with an average cumulative total of 13 sequences (range: 6–88 total sequences).



Phylum	Number of Taxa Depleted	Number of Taxa Enriched
Acidobacteria	129	8
Actinobacteria	292	74
AD3	2	0
Armatimonadetes	3	28
Bacteroidetes	4	95
Chlamydiae	0	1
Chloroflexi	65	25
Crenarchaeota	1	0
Cyanobacteria	1	6
Elusimicrobia	2	0
Fibrobacteres	0	2
Firmicutes	3	1
Gemmatimonadetes	31	1
Nitrospirae	6	0
Planctomycetes	235	38
α-Proteobacteria	66	209
β-Proteobacteria	22	101
δ-Proteobacteria	27	51
γ-Proteobacteria	0	12
Thermi	0	1
Verrucomicrobia	23	64
WPS-2	0	3
WS3	2	0
Unclassified	71	42
Total	**993**	**779**

FIG. 4. Number of taxa significantly enriched (*Avena* core microbiome) or depleted in the rhizosphere and their corresponding percent change in relative abundance (RA %). Data were aggregated at the phylum level after individual OTU analysis.

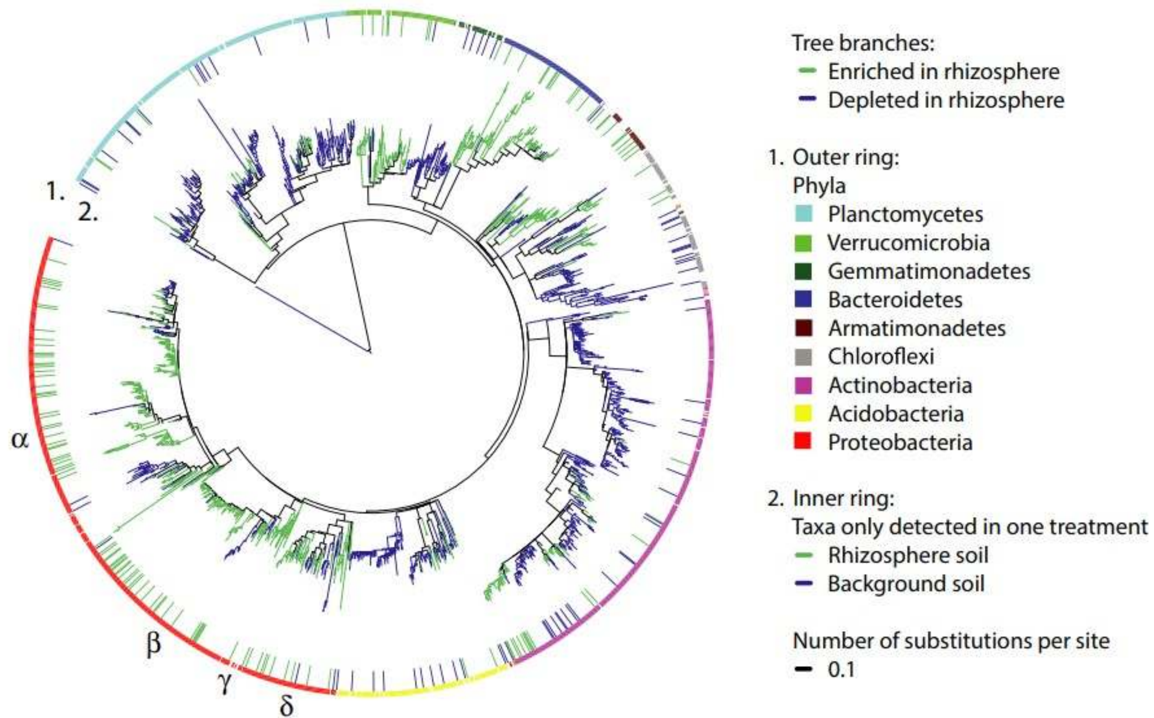


FIG. 5. Maximum likelihood tree for the *Avena* core microbiome (green branches) and taxa depleted in rhizosphere soil (blue branches), where the Ring 1 indicates OTU phyla, and Ring 2 indicates the 17% of taxa detected only in rhizosphere (green whiskers) or 7.6% detected only in background soils (blue whiskers). Proteobacterial classes are denoted by Greek letters.

Alpha-Proteobacteria and Actinobacteria were the dominant phyla in the rhizosphere and background soils (Appendix S1:Fig. S3). Relative to the background soil, the rhizosphere had lower relative abundances of Actinobacteria and higher relative abundances of alpha-Proteobacteria (Fig. 4, Appendix S1: Fig. S3). The rhizosphere was significantly enriched in the alpha-Proteobacterial order Rhizobiales (average +3.9% increase in relative abundance), the Caulobacteriaceae family (+1.6%), and the Sphingomonadaceae family (+1.9%), as well as the beta-Proteobacterial family Comamonadaceae (+2.0%) and the phylum Bacteroidetes (+1%) (Appendix S1: Tables S3 and S4). The Actinobacterial families Microbacteriaceae (+1.9%) and Nocardioidaceae (+0.60%) were also significantly enriched in the rhizosphere. While Actinobacteria were overall abundant in rhizosphere soil, many Actinobacterial taxa were significantly depleted in the rhizosphere (average – 8.9% decrease in phylum relative abundance), in particular in the families Solirubrobacterales (−4.0%) and Gaiellales (−1.3%). The rhizosphere was also depleted in Acidobacteria (−2.8%), Planctomycetes (−3.0%), and Gemmatimonadetes (−0.48%).

The core community was significantly phylogenetically clustered according to both net relatedness (NRI) and nearest taxon indices (NTI), and this clustering is apparent in the core microbiome maximum likelihood tree (Fig. 5). The taxa that were enriched in the rhizosphere showed significant phylogenetic clustering in the deeper branches of the tree (NRI: 7.32), as

well as at the terminal branches (NTI: 17.5). The taxa that were depleted in the rhizosphere were also phylogenetically clustered, but only at the terminal branches of the tree (NRI: 0.489; NTI: 15.2).

Correlation with environmental variables

We determined which environmental variables (background soil characteristics, plant characteristics, and soil climatic conditions) most strongly correlated with the rhizosphere and background soil communities (Table 1; see Appendix S1: Table S2 for complete list of results). Gravimetric water content at the time of sampling had the strongest correlation with the rhizosphere community composition ($r^2 = 0.89$), and was also significantly correlated with the water content the week prior to sampling ($r^2 = 0.76$, Appendix S1: Table S2). After soil moisture, the rhizosphere community was highly correlated with the region where the soil was sampled, silt content, and 8-month soil temperature range. Soil pH was moderately correlated with the rhizosphere soil community in comparison to these other variables ($r^2 = 0.67$). The background soil community composition, on the other hand, correlated strongly with soil pH ($r^2 = 0.94$). After soil pH and soil moisture, the background soil was highly correlated with chemical and pedological characteristics: exchangeable cations Mg and K, CEC, clay content, and salinity (EC). The region where the soil was sampled did not correlate as strongly with the background soil communities ($r^2 = 0.40$). BIO-ENV identified the combination of environmental variables that best correlated with the rhizosphere communities, where soil moisture and clay explained 85% of the variance in the data. After removing the effects of soil moisture and clay using CAP analysis, the species of *Avena* explained 5.2% of the variability in the rhizosphere dataset, but was not significant (ANOVA: $F_{1, 12} = 1.2$, $n = 4$ (*A. fatua*), $n = 8$ (*A. barbata*), $P > 0.1$). In agreement with this data, *A. barbata* collected at Sedgwick in a larger study grouped with *A. fatua* at Sedgwick, rather than with Hopland or Sierra samples (Appendix S1: Fig. S2).

Table 1. Correlation of environmental variables with rhizosphere and background soil microbial communities in NMDS space.

Environmental Variable	Rhizosphere (r^2)	*P*	Background (r^2)	*P*
Gravimetric Moisture	**0.89**	***	**0.83**	***
Region[1]	**0.74**	***	**0.40**	*
Silt	**0.72**	**	**0.53**	*
Temperature range[2]	**0.69**	**	0.19	
pH	**0.67**	**	**0.94**	***
K	**0.64**	*	**0.65**	**
Minimum temperature[3]	**0.52**	*	0.45	†
CEC	0.48	†	**0.77**	**
Clay	0.47	†	**0.77**	***
Mg	0.38		**0.79**	**
EC	0.25		**0.61**	*

The r^2 values represent the strength of the correlation ($n = 12$).
[1]Categorical variable representing grassland of origin (Sedgwick, Sierra, Hopland).
[2]8-month temperature range (maximum minus minimum temperature from January – August).
[3]Minimum temperature 2-weeks prior to harvest.
Significance of a correlation is designated by bold text and the number of asterisks: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, †$P < 0.1$ (marginally significant).

Discussion

A central goal in microbial ecology is to determine the factors that regulate the establishment of microbial communities (Nemergut et al. 2013, Wang et al. 2013). The rhizosphere environment is shaped by the plant root in collaboration with the root microbiome, as well as the edaphic characteristics of the soil, the regional climatic conditions, and the interactions between these factors (Berg and Smalla 2009, Philippot et al. 2013). The goals of this study were to determine how local soil characteristics and the regional climate shape the rhizosphere of a wild annual grass, and to determine the relative strength of the rhizosphere on microbial community assembly compared to the pressures imposed by the local and regional environment. To accomplish this, we examined *Avena* spp. rhizosphere soils and their surrounding soils across three Mediterranean grasslands. In addition, since we observed that the rhizosphere communities were strikingly different from the background soil communities, we evaluated the phylogenetic relationships within the *Avena* core microbiome.

First, we established that our sampling protocol was sufficient to detect changes in rhizosphere microbial communities and determined that unconventionally small amounts of rhizosphere soil (0.005 g) were capable of generating reproducible microbial communities. We targeted *Avena* root systems enmeshed in natural mixed plant communities and thus had lower root recovery than would be expected from monoculture or greenhouse conditions. We suspect that the reproducibility of the communities detected is due to the fact that we sufficiently sampled a microhabitat using composite sampling. For example, Nicol et al. (2003) found that sampling 0.1 g of soil was comparable to sampling 1 g and 10 g if the soil was homogenized prior to sampling; however, if the same masses of soil were sampled randomly without homogenization, the community composition was

not consistent across samples. In the current study, we combined multiple roots from three locations within a site for a total of 38 cm of root. Based on cell count data from a previous study on the *Avena* rhizosphere, 0.005 g of soil contains approximately 2×10^7 bacterial cells (DeAngelis et al. 2009). Our work suggests that composite sampling of microhabitats may permit the use of smaller volumes of soil for microbial community analysis, and allowed us to use a sampling strategy that targets rhizosphere communities immediately impacted by *Avena* roots.

We initially hypothesized that soil characteristics would be the main factor influencing rhizosphere microbial community assembly across the diverse soil types sampled in this study. However, we found that rhizosphere communities were more similar to each other than to the background soil communities from which each was derived, even across hundreds of kilometers; that is, the habitat sampled at each site (rhizosphere or background soil) accounted for the main source of variance in microbial community composition. While the strength of the rhizosphere effect is hypothesized to be plant specific (Bulgarelli et al. 2013), the magnitude of the rhizosphere effect is also hypothesized to depend on other factors, such as the length of time the plant has resided in the soil, or the soil management history (Berg and Smalla 2009, Philippot et al. 2013). Natural or low management systems are hypothesized to generate stronger rhizosphere effects (Philippot et al. 2013). The current study was situated in grasslands that have been uncultivated for the previous 50–60 yr; *Avena* has been a resident of these communities throughout this time period (Clegg and Allard 1972). *Avena* also has a number of selective mechanisms by which it could influence microbial community assembly. Relatives of *Avena* spp. produce avenacins, which are anti-microbial triterpenes that protect the plant against soil-borne diseases (Papanikolaou et al. 2010). *Avena* also effectively competes for N with native plants in N-limited grasslands, which could reflect a competitive belowground strategy for nutrient acquisition (Bulgarelli et al. 2013, Vacheron et al. 2013). The *Avena* root microbiome maintains high rates of N mineralization (Herman et al. 2006), chitinase and protease activities (DeAngelis et al., 2008), and nitrification rates (Hawkes et al. 2005) relative to the surrounding soil. *Avena* has also been shown to modify the arbuscular mycorrhizal fungi (AMF) populations of neighboring native grasses (Hawkes et al. 2006), which could alter soil nutrient availability (Nuccio et al. 2013).

However, within the rhizosphere and surrounding soil microbial communities, approximately half of the variation was due to the local soil environment and region in which the plants were sampled (21% and 22%, respectively). To determine how local soil and regional climatic conditions influenced microbial community assembly in these two habitats, we correlated the microbial communities with chemical, physical, and climatic characteristics measured on the background soil. We found that the rhizosphere communities correlated with a different set of environmental characteristics than the

communities in soil surrounding the root, which suggests that different factors shape these neighboring communities. Previous work has shown that pH correlates strongly with soil microbial community composition (Fierer and Jackson 2006). As predicted, the background soil communities correlated most strongly with soil pH, and were less correlated with other soil-specific factors (soil moisture, Mg, K, CEC, and clay content). The base cations Mg, K, and estimated CEC depend in part on soil pH (Essington 2003), and represent the short term store of nutrients available for microbial uptake (Bardgett 2012).

In contrast, the rhizosphere soil communities were only moderately correlated with the pH of the background soil, and correlated most strongly with factors relevant to the region and climate (soil moisture, region, 8-month range in soil temperatures, and silt). It is well known that plants are not passive agents in soil, and can substantially alter the soil microenvironment surrounding the root by changing substrate availability (Fierer et al. 2007), changing soil pH (Hinsinger et al. 2003, Blossfeld et al. 2013), and exuding chemoattractants and chemorepellents (Doornbos et al. 2012). Our analysis indicates that the moisture and temperature of the surrounding soil, and possibly the plant's response to these variables, was a stronger factor shaping the rhizosphere community than the edaphic characteristics poised by the background soil. We note that the rhizosphere communities clearly separated by grassland, while the background soil communities overlapped (Fig. 3A). The clear differences in the rhizosphere microbial communities may be due in part to plant interactions with local climatic conditions. Peiffer et al. (2013) speculated that a shared climate might have caused some similarities in rhizosphere responses among three fields with differing soil types. While climate change has been shown to alter the composition of plants across landscapes (Kelly and Goulden 2008), it is unclear how much of an impact this may have on associated soil microbial communities. Our results suggest that soil microbial communities may be more influenced by climatic conditions when in contact with a plant host.

Within the rhizosphere communities, it is intriguing that 17% of the bacteria that were consistently detected in the *Avena* core microbiome were not detected in the surrounding soils, even with robust levels of sequencing (ca. 70,000 sequences per sample), and despite the fact we could not collect true bulk soil in these densely rooted grasslands. We suspect that these taxa are present in the surrounding soil, but due to the ecology of the environment studied, they are likely present in low abundances and appear in the sequencing dataset only due to enrichment in the rhizosphere environment. In grassland soils dominated by annual plants, the rhizosphere is a transient microenvironment that lasts during the lifetime of a root, unlike perennial environments where the rhizosphere persists between seasons. Over decades of growing seasons, it is thought that the majority of the top 10 cm of soil has been in contact with roots at some point in time, and that background soil was probably rhizosphere soil in previous years. Tradeoff

theory predicts that traits which confer a selective advantage in one environment may cause a selective disadvantage in another environment (Futuyma and Moreno 1988, Sachs et al. 2009), which predicts that traits that make organisms successful competitors in the rhizosphere could make them poor competitors in background soil. This prediction raises a number of interesting questions about the evolutionary history of rhizosphere organisms in soil, such as the role of plant roots in generating and maintaining microbial species diversity, as well as microbial strategies for surviving in the absence of plant roots. Rhizobia, for example, are known to persist for years in soil in the absence of a host plant (Denison and Kiers 2004). Members of the beta-Proteobacteria and Bacteroidetes have been hypothesized to contain lineages of opportunistic (*r*-strategist) bacteria, which flourish in the presence of abundant labile carbon (Fierer et al. 2007). Less is known about the lifestyles of other taxa detected in this study that have few cultured representatives, such as the Armatimonadetes (formerly OP10) (Dunfield et al. 2012), or the diverse phylum Verrucomicrobia (Nunes da Rocha et al. 2011).

Since the rhizosphere soils were strikingly similar, even across hundreds of kilometers, we evaluated the phylogenetic relationships within the *Avena* core microbiome. This core microbiome is consistent with a previous study of *Avena* based on high-density microarray analysis (DeAngelis et al. 2009). Interestingly, the *Avena* rhizosphere is enriched in Microbacteriaceae, which has only been observed previously in the barley root microbiome, and not in the rhizospheres of other grasses (maize, wheat) or Arabidopsis (Bulgarelli et al. 2015). Across these grasslands, the *Avena* core microbiome showed strong evidence of rhizosphere-competent bacterial lineages according to the metrics NRI and NTI. NRI quantifies phylogenetic clustering in older phylogenetic lineages, while NTI quantifies clustering in groups that have diverged more recently in evolutionary history (Webb et al. 2002). Taxa in the *Avena* core microbiome are strongly clustered in both older and more recently diverged lineages, which suggests that these organisms have traits that confer rhizosphere competence which have been phylogenetically conserved. As plants only moved onto land approximately 450 million years ago (Sanderson et al. 2004), it is possible that the deeply rooted lineages represent adaptions to high-substrate, opportunistic lifestyles that originated independently of the rhizosphere. Indeed, some rhizosphere lineages appear to be conserved across multiple plant species (Bulgarelli et al. 2013, Schlaeppi et al. 2014), including plants that diverged 200 mya (Bulgarelli et al. 2015). Bulgarelli et al. (2015) hypothesized that conserved plant traits select for these shared consortia. The phylogenetic clustering detected in our study provides an additional hypothesis, where some of these bacterial lineages may have acquired rhizosphere competence before these plants diverged. We also observed clustering in the more recently diverged lineages, which may include relatively recent adaptations to the rhizosphere, such as group- or host-specific lineages, as well as adaptations to the local

environment. NRI and NTI can indicate selective community assembly processes (Stegen et al. 2012), where in this case the rhizosphere could act as a habitat filter because the soil environment is substantially altered surrounding a root (Hinsinger et al. 2003, Uren 2007, Berg and Smalla 2009). Additional opportunities for community selection events exist in Mediterranean annual grasslands, where the lifespan of annual plants is restricted to the wet winter and spring months (Jackson et al. 1988), and a large proportion of aboveground biomass senesces each year. In this ecosystem, drought-adapted and rhizosphere-adapted bacteria can flourish at different times of the year (Cruz-Martínez et al. 2009, Barnard et al. 2013). In this sense, the rhizosphere habitat in annual grasslands is ephemeral, and may play an important role in creating bacterial diversity in annual grassland soils.

In conclusion, we found that rhizosphere soils were more similar to each other than to the background soils from which they were derived, and the rhizosphere communities were more influenced by factors related the regional climate (soil moisture and temperature) than the background soil (primarily pH and pedological characteristics). We detected a number of organisms in the core microbiome that were not detectable in the background soil, which tradeoff theory would predict are rhizosphere specialists that are poor competitors in the background soil. Furthermore, taxa across multiple grasslands converged to a characteristic *Avena* core microbiome that was strongly phylogenetically clustered. Together, these results support the hypothesis that roots are important agents for the creation and maintenance of bacterial diversity in soil, and suggest that climate change has the potential to alter the rhizosphere microbiome.

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