Key microbial taxa in the rhizosphere of sorghum and sunflower grown in crop rotation

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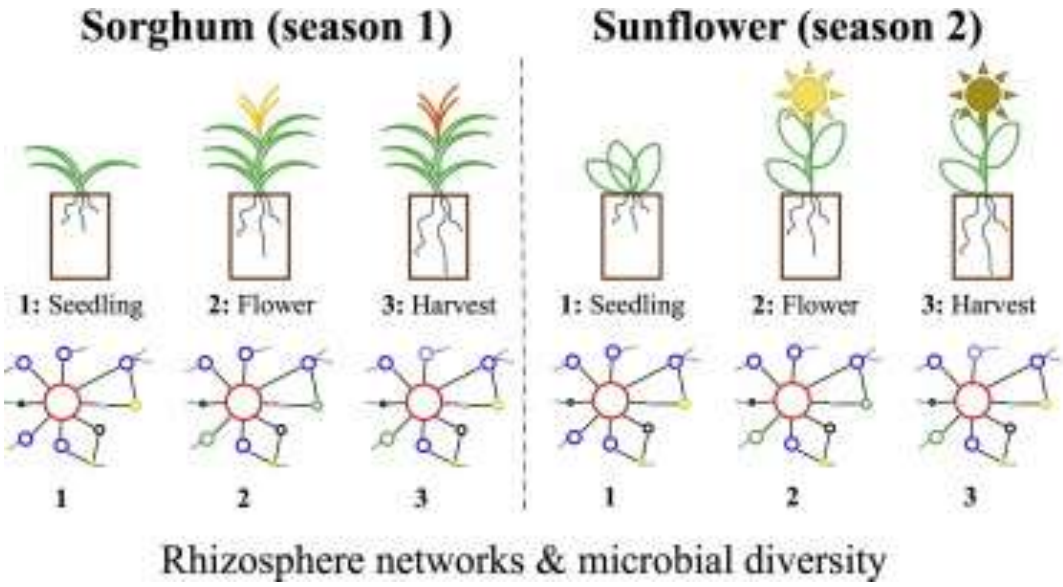
Highlights

- Rhizosphere microbial diversity and composition changed over time.
- Changes were due to both plant development stage and seasonality in bulk soil biota.
- Proteobacteria and Nitrospirae were overrepresented in the rhizosphere.
- Rhizosphere networks contained keystone taxa.
- Findings may contribute to microbial-based strategies to enhance crop productivity.

Graphical abstract



ABSTRACT

Microbes are key determinants of plant health and productivity. Previous studies have characterized the rhizosphere microbiomes of numerous plant species, but little information is available on how rhizosphere microbial communities change over time under crop rotation systems. Here, we document microbial communities in the rhizosphere of sorghum and sunflower (at seedling, flowering and senescence stages) grown in crop rotation in four different soils under field conditions. A comprehensive 16S rRNA-based amplicon sequencing survey revealed that the differences in alpha-diversity between rhizosphere and bulk soils changed over time. Sorghum rhizosphere soil microbial diversity at flowering and senescence were more diverse than bulk soils, whereas the microbial diversity of sunflower rhizosphere soils at flowering were less diverse with respect to bulk soils. Sampling time was also important in explaining the variation in microbial community composition in soils grown with both crops. Temporal changes observed in the rhizosphere microbiome were both plant-driven and due to seasonal changes in the bulk soil biota. Several individual taxa were relatively more abundant in the rhizosphere and/or found to be important in maintaining rhizosphere microbial networks. Interestingly, some of these taxa showed similar patterns at different sampling times, suggesting that the same organisms may play the same functional/structural role at different plant growth stages and in different crops. Overall, we have identified prominent microbial taxa that might be used to develop microbiome-based strategies for improving the yield and productivity of sorghum and sunflower.

Keywords: Microbial networks; Soil; Field conditions; Diversity; Composition.

1. INTRODUCTION

Rhizosphere microbial communities play key roles in determining plant health and productivity (Philippot et al., 2013). For instance, mycorrhizal fungi and nitrogen-fixing bacteria are responsible for 40-50% of all nitrogen (Udvardi and Poole, 2013), and up to 75% of phosphorus (van der Heijden et al., 2008), that is acquired by plants annually. Thus, the manipulation of the rhizosphere microbiome has the potential to improve the yield of agronomically important crops (Turner et al., 2013).

Previous research has shown that soil type is a major determinant of rhizosphere microbial communities, most likely as a result of the different microbial "inocula" present in each soil type (Bulgarelli et al., 2012; Edwards et al., 2015; Lundberg et al., 2012; Peiffer et al., 2013; Schlaeppi et al., 2014). Other factors such as climatic conditions, plant species, plant development stage and the interactions between all these factors are also drivers of microbial community composition (Edwards et al., 2015; Mendes et al., 2014; Peiffer et al., 2013; Zarraonaindia et al., 2015). For example, a study of the rhizosphere microbiota of three model plants (*Arabidopsis*, *Medicago* and *Brachypodium*) and three crops (*Brassica*, *Pisum* and *Triticum*) showed that all plant species tested had very different microbiota from each other and from unplanted soil, and that *Arabidopsis* had the weakest influence on its microbiota (Tkacz et al., 2015). The authors also revealed that although plants posed strong selection on rhizosphere microbial communities, their composition and stability also depended on soil type. Shi et al. (2015) found that the rhizospheric bacterial communities of *Avena fatua* changed as plants grew and that the pattern of temporal succession was consistent and repeatable over two growing seasons. In all, soil type, together with agricultural practices that directly influence the soil, are thought to be the main drivers of rhizosphere microbial communities in agricultural settings,

while climate conditions and plant species are the most influential factors in the natural ecosystems (Philippot et al., 2013).

Plant roots are thought to select for specific microbes by producing an array of metabolites, including secondary metabolites such as antimicrobial compounds (Bais et al., 2006) and defence phytohormones (Lebeis et al., 2015), that vary with plant species and plant age (Lynch and Whipps, 1990). Roots also create a distinct soil microhabitat by altering the pH and oxygen concentrations in the soil surrounding the root (Hacquard et al., 2015). However, although our understanding of rhizosphere microbial communities has recently improved, many aspects of the assembly of these communities are not well understood (van der Heijden and Schlaeppi, 2015), especially for plants growing under field conditions. Most rhizospheric studies are performed on plants grown in controlled environments, with comparatively less studies conducted in the field (e.g., Edwards et al., 2015; Peiffer et al., 2013; Rascovan et al., 2016). It has been demonstrated that the small containers used in most indoor experiments have a negative impact on root function, root distribution and plant growth (Poorter et al., 2012).

In this study, we investigated the diversity and composition of the prokaryotic (bacterial and archaeal) communities in the rhizosphere of two economically important crop species, sorghum and sunflower, grown in crop rotation under field conditions in South Africa. Soil microbial communities were analysed at pre-planting and at three plant growth stages (i.e., seedling, flowering, and senescence) using Illumina MiSeq sequencing of 16S rRNA gene amplicons. The two crops were grown in four different soils located in two farms (two soils each) approximately 300 km apart. Our primary goals were: (i) to investigate how the diversity (alpha and beta components) of the soil prokaryote community associated with the roots of sorghum and sunflower grown under crop rotation change with plant development stage and soil properties

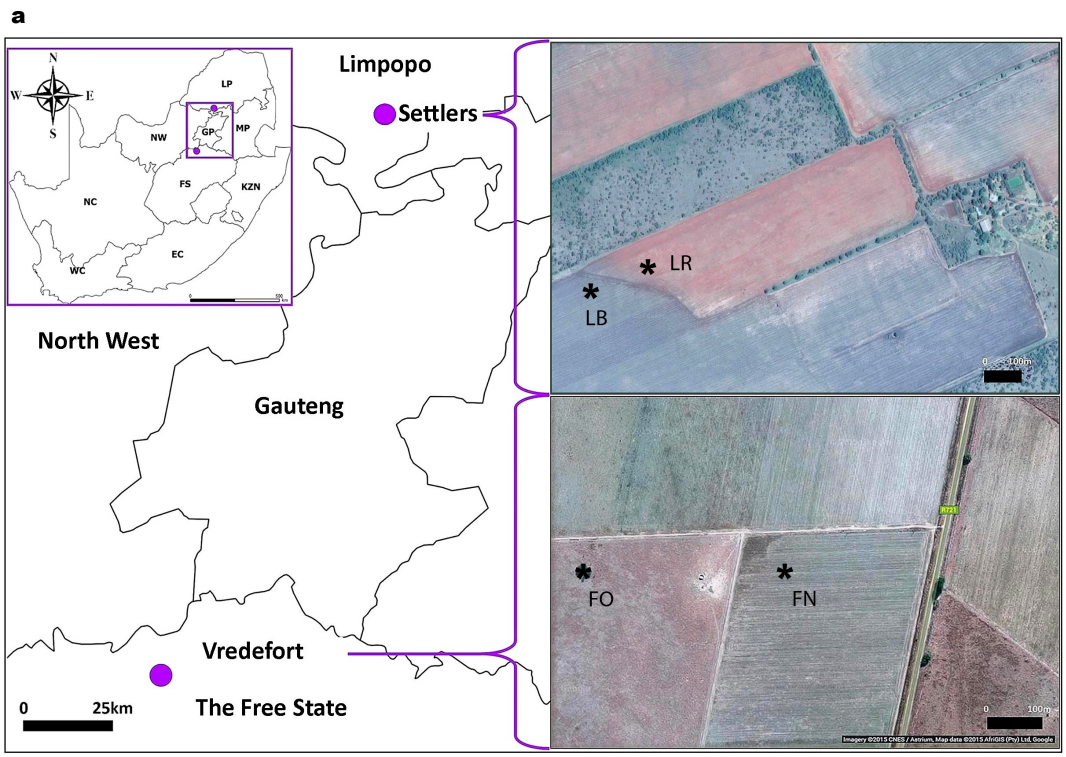
and (ii) to determine whether the rhizosphere of these two contain stable "key" microbial community members. Stable key rhizosphere microbes hold great potential to influence the host phenotype (Busby et al., 2017) and could ideally be used to improve crop yield and productivity.

2. MATERIALS AND METHODS

2.1. Field sites and sample collection

The four fields were located in two farms (two fields each), situated approx. 300 km apart, near Settlers (Limpopo province, 27°02'43.4" S, 27°23'46.6" E) and Vredefort (Free State province, 24°57'09.7" S, 28°24'01.4" E) in South Africa (Figure 1a). Settlers had a mean annual temperature (MAT) of 19.1ºC and a mean annual precipitation (MAP) of 606mm during the last ten years. Vredefort had a MAT of 16.6 and a MAP of 639 mm in the same period of time. The four fields were cropped with sorghum and sunflower cultivars two years before the start of the study.

Soil sampling extended over two consecutive seasons, from 19 November 2014 to 3 March 2016, (Figure 1b). In the first season the fields were planted with sorghum (cultivar K2) and in the second with sunflower (cultivar PAN 2057). Similar conventional culture practices were used in all fields, including ammonium nitrate-based fertilization, weed control, and pest control. Sampling was performed at pre-planting and three different stages of growth (seedling, flowering and senescence stages) for each of the two crops grown in rotation. Bulk soil was collected prior to planting. For all remaining time points paired rhizosphere and bulk soil samples were collected. Rhizosphere soils were collected by uprooting the plants and recovering the soil firmly attached to the roots. Bulk soils were retrieved from loose soil within the field and therefore subjected to the same agriculture practices than the respective rhizosphere soil. Bulk soils



Growth stage	Pre-planting	Seedling	Flowering	Harvesting	Seedling	Flowering	Harvesting
Time point	1	2	3	4	5	6	7
Sample type	Bs	Bs + Rs	Bs + Rs	Bs + Rs	Bs + Rs	Bs + Rs	Bs + Rs
#samples	20	20 + 20	20 + 20	20 + 20	20 + 20	20 + 20	20 + 20

Figure 1. Map of the study region in South Africa (a) and experimental design (b). Asterisks denote the different soil types sampled. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old.

contained root debris from the same crops planted in the previous two years. For each growing stage, five sites were selected along a 50 m transect in each of the four fields. At each of the five sites, three rhizosphere and three bulk soil samples were aseptically collected and homogenised in sterile plastic bags to obtain a single composite sample. For molecular analysis, aliquots of soil samples were stored at -80°C until further processing. Overall, 260 samples were collected over two consecutive growing seasons (4 fields x 2 crops (sorghum and sunflower) x 3 growing stages (seedling, flowering and senescent) x 2 habitat types (rhizosphere and bulk soil) x 5 replicates + 20 (4 fields x 5 replicates) pre-planted bulk soil).

2.2. Soil nutrient analysis

Analysis of both rhizosphere and bulk soil samples was conducted at Bemlab (SANAS Accredited Testing Laboratory, Somerset West, South Africa) using standard procedures. Prior to analysis, soil samples were sieved (2 mm) and dried overnight at 50 °C. The slurry technique was used to measure pH (1:3 soil/deionised water) with a Crison Bench pH meter (Crison Instruments, Barcelona, Spain) after allowing soil to settle for 30 min. Total C and N were determined using a Truspec elemental determinator (LECO, USA). Total P was measured using the P Bray method. Ammonium acetate extraction was used to measure salt concentrations (K^+, Ca^{2+}, Mg^{2+}) using inductively coupled plasma atomic emission spectroscopy (ICP-OES; Spectro Genesis, Spectro Analytical Instruments GmbH, Germany).

2.3. DNA extraction and 16S rRNA amplicon sequencing

Total DNA was extracted from 0.5 g soil using a MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. PCRs were

performed in a single-step PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) with primer pairs 515F (5′-GTGYCAGCMGCCGCGGRA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAG-3′), which amplify both bacteria and archaea. PCR products from all samples were quantified using the PicoGreen dsDNA assay, pooled together in equimolar concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was carried out on an Illumina MiSeq 2000 using a paired-end approach at the Molecular Research LP next generation sequencing service (http://www.mrdnalab.com).

2.4. Data processing and statistical analysis

Raw Illumina sequence data were processed using QIIME v1.8.0 (Caporaso et al., 2010b). Briefly, demultiplexing and quality filtering was performed with the default parameters and a phred offset of 33. Chimera identification and filtering was performed with the Greengenes database (gg_13_8) with the usearch61 algorithm using default parameters. The remained sequences were binned into operational taxonomic units (OTUs) at a 97% sequence similarity cut-off using an open reference process with uclust: amplicon sequences were first clustered against the Greengenes database (gg_13_08) and sequences that did not match with the reference sequence collection were clustered *de novo*. Representative sequences were aligned with PyNAST (Caporaso et al., 2010a). Taxonomy was assigned with the uclust classifier with the Greengenes 13_8 reference database. A phylogenetic tree built with FastTree and an OTU table generated containing both bacteria and archaea. Only OTUs present in at least 10 samples and represented by at least 3 sequences were retained in the final OTU table.

Faith's phylogenetic diversity (PD), richness, Chao1 and Good's coverage estimators were calculated using QIIME. Differences in PD, richness and Chao1 estimator between the different soils at pre-planting were assessed using Wilcoxon-Mann-Whitney *post hoc* tests after ensuring that an overall Kruskal-Wallis test was significant.

To determine the factors which explained differences in alpha-diversity (PD, richness and Chao1 indexes), soil chemistry and beta-diversity (top two axes of weighted UniFrac distance matrices; Lozupone and Knight, 2005) after planting, we applied linear mixed models. In those models, habitat type (rhizosphere, bulk soil) and growth stage (seedling, flowering, senescence) were fixed factors, whereas soil type was considered a random factor. All models were fitted using the lme4 package (Bates et al., 2015). Alpha-diversity and soil chemistry differences were tested on least square means using Tukey contrasts with the lsmeans package. For beta-diversity the statistical significance of fixed predictors was assessed using Type III ANOVA with the Satterthwaite approximation to obtain the degrees of freedom and of random effects using a likelihood ratio test, both using the package *lmerTest* (Kuznetsova et al., 2016).

Pearson correlations were used to identify relationships between soil properties and alpha-diversity. The relationships between changes in community composition (beta-diversity) and soil chemistry variables were explored using BIO-ENV (Clarke and Ainsworth, 1993). BIO-ENV uses the Spearman rank correlation coefficient (ρ) to determine the degree of association with community variability. Abiotic data were standardized (mean=0, standard deviation=1) and pair-wise distances computed based on Euclidean distances. Pair-wise distances were visualised using principal coordinate analysis (PCoA).

To identify the key OTUs in the sorghum and sunflower rhizosphere, we used two different approaches. DESeq2 (Love et al., 2014) was used to detect microbial OTUs which were

differentially abundant in the rhizosphere *vs* bulk soil communities at the different stages of growth. Soil type was included in the model as a random factor to remove soil batch effects. DESeq2 has been shown to be efficient in testing microbiome data (DiGiulio et al., 2015; Wagner et al., 2016). Secondly, we used network analysis, following the methodology described by Shi et al. (2016), to identify OTUs (nodes) that potentially play structural roles in rhizosphere networks at the different stages of growth. The analysis is based on the concepts of within-module connectivity (*Zi*) and between-module connectivity (*Pi*) (Guimerà and Amaral, 2005), which separate nodes into four categories: (i) module hubs (highly connected OTUs within modules, $Zi > 2.5$), (ii) network hubs (highly connected OTUs within the entire network, $Zi > 2.5$ and $Pi > 0.62$), (iii) connectors (OTUs that connect modules, $Pi > 0.62$) and (iv) peripherals (OTUs connected in modules with few outside connections, $Zi < 2.5$ and $Pi < 0.62$). Module hubs, connectors and network hubs have been proposed to be putative keystone taxa (Shi et al., 2016); that is, taxa that are critical in maintaining community structure and function. These analyses were performed using the Molecular Ecological Network Analyses (MENA) pipeline (Deng et al., 2012) available at http://ieg2.ou.edu/MENA/. Refined taxonomy assignment of key OTU representative sequences was performed using EzBioCloud server (http://www.ezbiocloud.net) (Yoon et al., 2017).

For downstream applications that can be influenced by unequal sampling depth (such as, diversity indexes, PCoA, linear mixed models, network analysis) we rarefied the OTU table, as recommended by Weiss et al. (2017) after McMurdie and Holmes (2014). Rarefaction was done at a depth of 16,284 sequences per sample, the lower number of sequences in a given sample. For DESeq, raw (un-rarefied) OTU counts were used (DiGiulio et al., 2015). Statistical significance

was assessed at α=0.05 and where applicable, *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate.

3. RESULTS AND DISCUSSION

Sequence analysis of the full data set (n=260) yielded 46,079 OTUs comprising 5,690,871 quality sequences (16,284-34,007 sequences per sample). After rarefaction, a total of 45,510 OTUs and 4,233,840 sequences remained. Good's coverage (range 83-98%, average 86.8%) estimates indicated reasonable sequencing coverage (Supplementary data Table S1); although a larger volume of sequences will be required to comprehensively sample the diversity of these communities, especially for sorghum soils at seedling (Supplementary data Figure S1). Indeed, it has been shown that it is highly unlikely to cover the total diversity of both bacteria and archaea using a single primer set (Klindworth et al., 2012).

3.1. Soil type shapes microbial community diversity at pre-planting

We found that the four soils differed in chemistry (Supplementary data Table S2 and Figure S2), and in alpha diversity (Supplementary data Figure S3) at pre-planting. Differences in microbial community composition (β-diversity) evaluated using weighted UniFrac distances were also observed (Supplementary data Figure S4), indicating that each soil type harboured a different microbial "start" inoculum. Altogether, these results indicate that the soils differed both in alpha and beta diversity at the beginning of the study. Microbial taxa specific to soil type could have resulted from, for example, limited dispersal between the two farms (ca. 300 km apart) and/or local sorting mechanisms that completely exclude or enrich some microbial taxa in a given soil type (Hanson et al., 2012). Previous work on plant associated microbes has shown that soil type

is a strong determinant of microbial diversity (Bulgarelli et al., 2012; Edwards et al., 2015; Lundberg et al., 2012; Peiffer et al., 2013). Nevertheless, teasing apart the effect of soil type on microbial community composition remains challenging under field conditions as not only the soil characteristics influence the soil microbiome, but also the climate, the cropping history or the agricultural management (Schreiter et al., 2014 and references therein).

3.2. Habitat type and sampling time influence post-planting microbial community diversity

Soil and habitat type (rhizosphere and bulk soil) were significant predictors of microbial alpha-diversity in sorghum soils (Table 1). In contrast, alpha-diversity in sunflower fields was only determined by soil type. Overall, alpha-diversity was higher in sorghum compared to sunflower fields (Supplementary data Figure S5). This effect was driven by higher diversity of sorghum rhizosphere soils at flowering and senescence in conjunction with a decrease in diversity in sunflower rhizosphere soils at flowering (Figure 2). Both habitat type and sampling time (plant growth stage) were important in explaining differences in beta-diversity (Table 2), implying that the temporal changes observed in the rhizosphere microbiome were probably both due to temporal changes in the bulk soil biota and plant-driven. Temporal variability of bulk soil microbial communities is a well-established phenomenon (Lauber et al., 2013) that may result in changes in the rhizosphere microbiome, since bulk soil communities act as a source for rhizosphere communities (Zarraonaindia et al., 2015). Variations between plant species in the quantity and quality of rhizodeposits (Vančura, 1964), which also change over time (Miller et al., 1990), may explain the contribution of the plant to these patterns. Differences between the two plant species in their efficiency in nutrient acquisition, which in turn may alter the nature of

Table 1. Factors predicting alpha-diversity in soil bacterial communities.

	Sorghum			**Sunflower**		
	PD	**Richness**	**Chao1**	**PD**	**Richness**	**Chao1**
R^2	0.55	0.31	0.25	0.59	0.42	0.51
Growth stage (GS)	$F_{2,116}$=1.40 P=0.25	$F_{2,116}$=2.51 P=0.08	$F_{2,116}$=2.73 P=0.07	$F_{2,116}$=3.12 P=0.48	$F_{2,116}$=0.93 P=0.39	$F_{2,116}$=3.33 ***P*<0.05**
Habitat type (HT)	$F_{1,116}$=14.42 ***P*<0.001**	$F_{1,116}$=4.17 ***P*<0.05**	$F_{1,116}$=5.13 ***P*<0.05**	$F_{1,116}$=0.53 P=0.47	$F_{1,116}$=0.91 P=0.34	$F_{1,116}$=3.37 P=0.07
GS x HT	$F_{2,116}$=1.34 P=0.27	$F_{2,116}$=0.96 P=0.38	$F_{2,116}$=1.22 P=0.29	$F_{2,116}$=2.12 P=0.12	$F_{2,116}$=2.21 P=0.11	$F_{2,116}$=3.53 ***P*<0.05**
Soil type	χ^2_1=68.2 ***P*<2e-16**	χ^2_1=24.4 ***P*<8e-7**	χ^2_1=68.2 ***P*<1e-4**	χ^2_1=83.2 ***P*<2e-16**	χ^2_1=46.5 ***P*<9e-12**	χ^2_1=83.2 ***P*<2e-16**

Statistics describe linear mixed models of alpha-diversity. Significance was assessed using type III ANOVA with F tests for fixed effects and likelihood ratio tests for random effects. Bold values indicate statistically significant (P<0.05) results after correction for multiple comparisons using the Benjamini-Hochberg false discovery rate. PD, Faith's phylogenetic diversity.

Table 2. Factors predicting beta-diversity of soil bacterial communities

	Sorghum		**Sunflower**	
	PCo1	**PCo2**	**PCo1**	**PCo2**
R^2	0.87	0.26	0.81	0.34
Growth stage (GS)	$F_{2,116}$=3.12 ***P*=0.047**	$F_{2,116}$=6.77 ***P*=0.0016**	$F_{2,116}$=16.90 ***P*=3.6e-7**	$F_{2,116}$=4.69 ***P*=0.011**
Habitat type (HT)	$F_{1,116}$=12.83 **P=0.0005**	$F_{1,116}$=4.13 **P=0.044**	$F_{1,116}$=7.8 **P=0.0061**	$F_{1,116}$=10.41 **P=0.0016**
GS x HT	$F_{2,116}$=0.76 P=0.467	$F_{2,116}$=0.045 P=0.955	$F_{2,116}$=0.37 P=0.68	$F_{2,116}$=0.089 P=0.914
Soil type	χ^2_1=215 **<2e-16**	χ^2_1=12.4 ***P*=4e-4**	χ^2_1=169 **<2e-16**	χ^2_1=23.2 ***P*=1e-6**

Statistics describe linear mixed models of weighted UniFrac principal coordinates. Significance was assessed using type III ANOVA with F tests for fixed effects and likelihood ratio tests for random effects. Bold values indicate statistically significant (P<0.05) results after correction for multiple comparisons using the Benjamini-Hochberg false discovery rate.

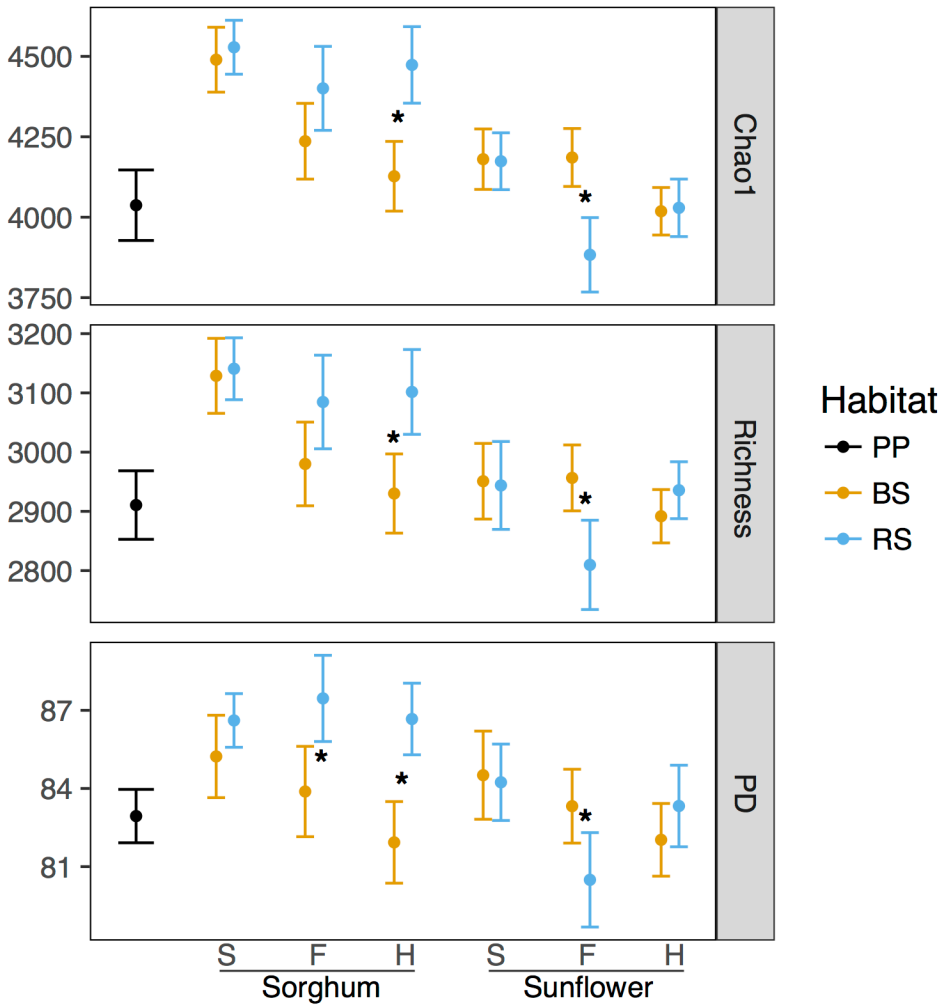


Figure 2. Changes in alpha-diversity measured using Faith's phylogenetic diversity (PD), richness and Chao1 estimator. Least-square mean for each habitat type (rhizosphere and bulk soil) is plotted with ± 1 s.e of the mean. Asterisks indicate a significant habitat type effect (ANOVA $P < 0.05$) for a given sampling date. Values at pre-planting are also included for comparison.

nutrient competition between plants and microbes (Bardgett et al., 1999), are also likely to have a strong influence on the soil microbial community. Altogether, the influence of habitat type and growth stage was less important than the influence of soil type in explaining diversity patterns (Supplementary Figure S5). There are several contrasting reports in the literature relating to whether habitat type (rhizosphere *vs* bulk soil), plant grow stage, plant species or soil type is the dominant factor in driving biodiversity patterns in soil microbial communities (Berg and Smalla, 2009; Bulgarelli et al., 2015; Chaparro et al., 2014; Correa-Galeote et al., 2016; Donn et al., 2015; Edwards et al., 2015; Peiffer et al., 2013; Schreiter et al., 2014; Tkacz et al., 2015). These results support the concept that soil type is a major factor in shaping microbial community diversity and composition under field conditions, whereas habitat type and plant growth stage seem to have a relatively small impact on these communities. We hypothesized that the latter can be due to the fact that with our sampling strategy the separation between bulk and rhizosphere soil is not as obvious as in other studies, because, for example, a) bulk soils were not separated from rhizosphere soils using root-excluding mesh bags and b) bulk soils contained root debris from the same crops planted in the previous two years.

3.3. Soil parameters influencing post-planting microbial community diversity and composition

Soil chemistry differed between soil types (Table 3, Supplementary data Table S2) and crops (Supplementary data Figure S7), but not between rhizosphere and bulk soil (habitat type) for any of the two crops (Table 3). Furthermore, soil parameters did change across the different growth stages in sorghum soils (Table 3). For example, carbon levels were higher at flowering and senescence than at seedling, while nitrogen showed the opposite trend (Supplementary data

Table 3. Factors predicting soil chemistry

	Sorghum		**Sunflower**	
	PCo1	**PCo2**	**PCo1**	**PCo2**
R^2	0.54	0.45	0.93	0.85
Growth stage (GS)	$F_{2,116}$=45.61 ***P*=2.4e-15**	$F_{2,116}$=2.48 *P*=0.08	$F_{2,116}$=0.26 *P*=0.76	$F_{2,116}$=0.42 *P*=0.65
Habitat type (HT)	$F_{1,116}$=0.06 P=0.80	$F_{1,116}$=0.03 P=0.85	$F_{1,116}$=2.25 P=0.13	$F_{1,116}$=0.01 P=0.91
GS x HT	$F_{2,116}$=0.31 P=0.73	$F_{2,116}$=1.04 P=0.35	$F_{2,116}$=2.20 P=0.11	$F_{2,116}$=0.01 P=0.99
Soil type	χ^2_1=28.1 ***P*=1e-7**	χ^2_1=51.5 ***P*=7e-13**	χ^2_1=292 **<2e-16**	χ^2_1=199 **<2e-16**

Statistics describe linear mixed models of Euclidean distances principal coordinates. Significance was assessed using type III ANOVA with F tests for fixed effects and likelihood ratio tests for random effects. Bold values indicate statistically significant (P<0.05) results after correction for multiple comparisons using the Benjamini-Hochberg false discovery rate.

Table 4. Pearson correlation coefficients between soil properties and phylogenetic distance

	Sorghum		**Sunflower**	
	Rhizosphere	**Bulk soil**	**Rhizosphere**	**Bulk soil**
K	-	-	r=-0.54 *P*<0.001	r=-0.71 *P*<0.001
Ca	-	-	r=-0.58 *P*<0.001	r=-0.47 *P*<0.001
Mg	-	-	r=-0.52 *P*<0.001	r=-0.50 *P*<0.001
P	-	-	r=0.52 *P*<0.001	r=0.64 *P*<0.001
C	r=0.28 *P*=0.025	r=0.34 *P*=0.007	-	r=0.35 *P*=0.006
N	-	r=0.36 *P*=0.004	-	-

Only *P*-values <0.05 are indicated.

Figure S8). Phylogenetic diversity was significantly correlated with %C in sorghum and sunflower bulk soils in addition to sorghum rhizosphere soils, while %N correlated with PD only in sorghum bulk soils (Table 4). K, Ca, Mg and P levels were good predictors of PD patterns in sunflower soils but not in sorghum soils (Table 4). Similar results were found using other two alpha-diversity indexes (richness and Chao1 estimator) (data not shown). Using a BEST analysis we found that P and C levels had the strongest correlation with sorghum rhizosphere communities (ρ=0.21, P=0.001), whereas sorghum bulk soil communities were correlated with P, C and N (ρ=0.26, P=0.001). In sunflower fields, the combination of K, Ca, Mg, P and C better explained the variation of rhizosphere communities (ρ=0.38, P=0.001), while bulk soil communities were explained by K and P (ρ=0.51, P=0.001). Previous work has shown that factors such as P, C and K correlate strongly with soil microbial community diversity and composition (Lauber et al., 2008; Leff et al., 2015; Ranjard et al., 2013), but the results of this study show that a different set of chemical parameters shape rhizosphere and bulk soil microbial communities in both sorghum and sunflower fields.

3.4. Rhizosphere microbial communities are relatively stable over time

Rhizosphere microbial communities were dominated by bacteria of the phyla *Proteobacteria* (31.9% (mean) ± 6.0% (SD) of all reads, mainly *Alphaproteobacteria* (18.3% ± 4.2%)), *Actinobacteria* (20.4% ± 6.9%), *Bacteroidetes* (14.9% ± 6.6%) and archaea of the phylum *Crenarchaeota* (13.2% ± 7.0%) (Figure 3, Supplementary data Table S3). Members belonging to these three main bacterial phyla appear to be consistently associated with plant roots (Bulgarelli et al., 2012; Edwards et al., 2015; Lundberg et al., 2012; Peiffer et al., 2013). Much less is known about the abundance of archaea associated with plant roots, although they seem not to be

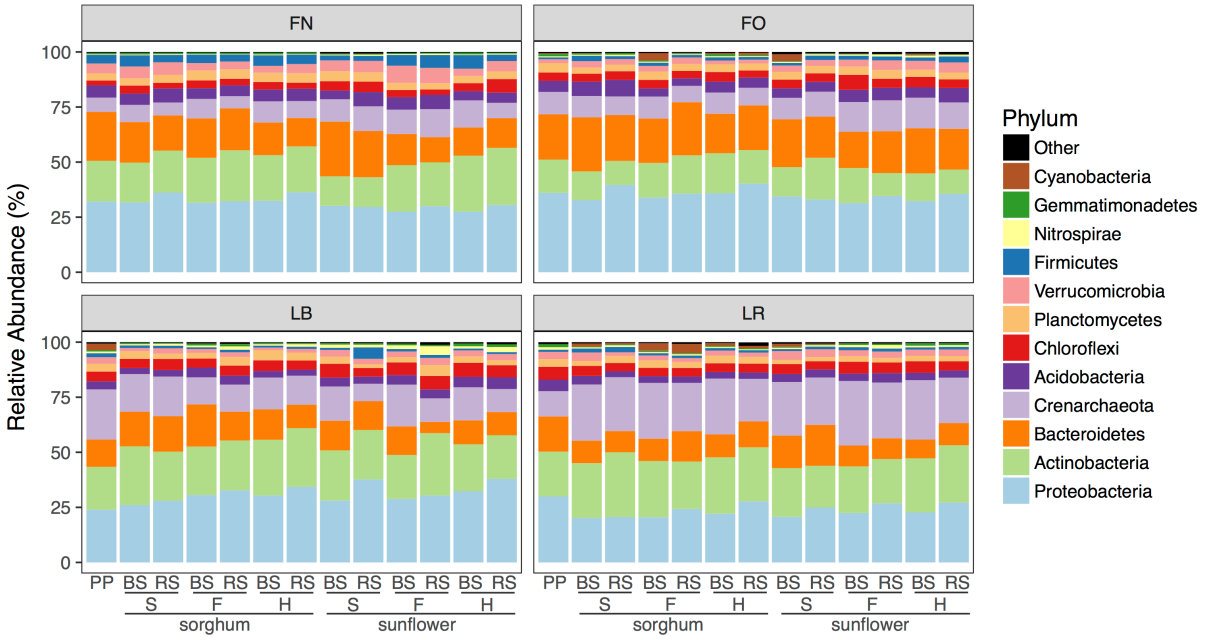


Figure 3. Relative abundance of major phyla (> 0.5% of reads) over time for each soil type. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old. PP, pre-planting; BS, bulk soil; RS, rhizosphere soil. S, seedling; F, flowering; H, harvest.

major inhabitants of the rhizosphere (Buée et al., 2009); but see Simon et al., (2000) for another exception. It is possible that the high abundance of archaea in this study is a consequence of the reverse primer used (909R), as most studies were performed with 806R as the reverse primer.

The relative abundance of most microbial phyla remained relatively stable in both the rhizosphere and bulk soil throughout the sampling period (Supplementary data Figure S9). Thus, the bacterial phyla *Proteobacteria* (in sorghum) and *Nitrospirae* (in sunflower) were the only two phyla enriched in the rhizosphere relative to the bulk soil at all stages of plant growth (Supplementary data Figure S10). Although it is unlikely that all members of a phylum would share common ecological characteristics, *Proteobacteria* have been suggested to respond positively to labile carbon compounds (r-strategists; Fierer et al., 2007), which are abundant in plant root exudates (Bais et al., 2006). Conversely, *Nitrospirae* are thought to be slow-growing (k-strategist) chemolithoautotrophs involved in nitrification (Daims et al., 2015). In contrast, the phylum *Crenarchaeota* (most belonging to the genus *Nitrososphaera*) was significantly reduced in the sunflower rhizosphere compared to the bulk soil (Supplementary data Figure S10). Interestingly, members of the genus *Nistrososphaera* can also perform nitrification (Offre et al., 2013). Nitrification can be either inhibited or stimulated in the rhizosphere (Philippot et al., 2013 and references therein), which can lead to changes in the availability of ammonium and nitrate in soils. Whether sunflower is increasing the abundance of nitrifying bacteria while decreasing the abundance of nitrifying archaea in the rhizosphere, relative to their respective abundances in the bulk soil, needs to be further investigated.

The relative abundance of the ten major families also remained relatively stable in both the rhizosphere and bulk soil through the sampling period (Supplementary data Figure S11). Of these, only the family *Hyphomicrobiaceae* (in sorghum and sunflower), *Gaiellaceae* and

Sphingomonadaceae (both in sunflower) were differentially more abundant in the rhizosphere than in the bulk soil (Supplementary data Figure S12). The families *Hyphomicrobiaceae* and *Sphingomonadaceae* were shown to be central in the rhizosphere of other plant species such as Arabidopsis and sugarcane (Yeoh et al., 2016), suggesting that these families may contain taxa critical to the function of rhizosphere microbial communities.

3.5. Key rhizosphere taxa

A total of 131 OTUs (representing 7.6% of the total number of reads in cultivated soils, n=240) were found to be more abundant in the rhizosphere compared to the bulk soil, while 90 (9.58% of the reads) showed the opposite trend (Supplementary data Table S4). Most OTUs overrepresented in the bulk soil belonged to *Bacteroidetes* (56 OTUs), *Proteobacteria* (17 OTUs, all *Alphaproteobacteria*) and *Actinobacteria* (8 OTUs). Of the highly abundant rhizospheric OTUs, a large majority (80 OTUs) belonged to *Proteobacteria* (29 *Alphaproteobacteria*, 28 *Betaproteobacteria*, 19 *Gammaproteobacteria* and 4 *Deltaproteobacteria*). *Bacteroidetes* (25 OTUs) and *Actinobacteria* (14 OTUs) were also overrepresented in rhizosphere soils. Interestingly, none of these 131 OTUs were found in seedling sunflower rhizospheric samples, indicating that two weeks after germination are enough to observe a rhizosphere effect in sorghum but not in sunflower plants. Furthermore, 29 of these OTUs (24 *Proteobacteria*, 2 *Bacteroidetes*, 1 *Actinobacteria*, 1 *Acidobacteria* and 1 *Verrucomicrobia*) were found in greater abundance in the rhizosphere compared to the bulk soil in at least two growth stages, the majority in both sorghum and sunflower rhizospheric soils (Figure 4, Supplementary data Table S5). This suggests that the same microbial taxa may be functionally important in the rhizosphere of different plant species and at different growth stages.

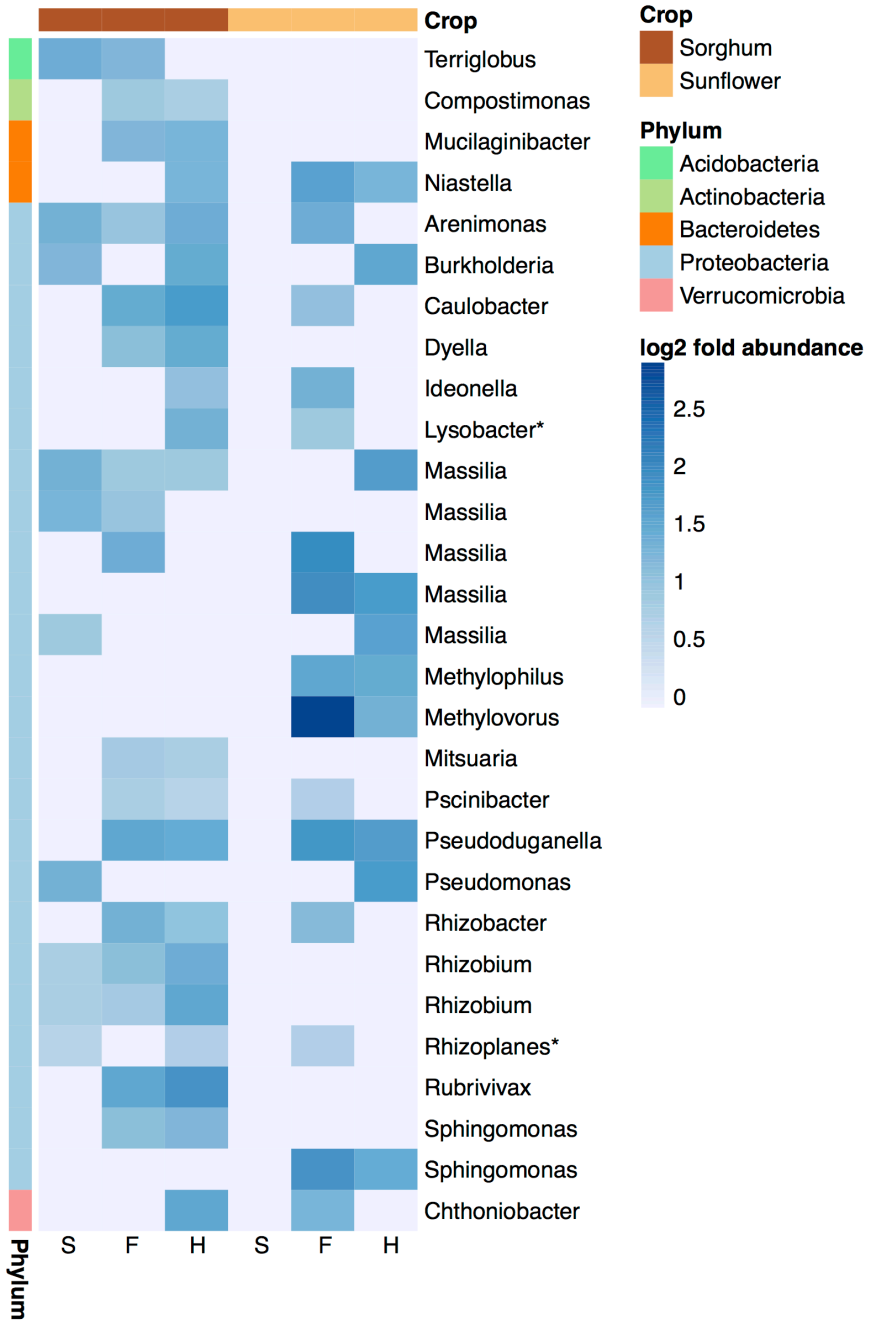


Figure 4. Enrichment of the 29 OTUs, found in at least two growth stages, in the rhizosphere compared with bulk soils as determined by differential abundance analysis using DESeq. Significance was assessed using a Likelihood ratio test at P <0.05. Asterisks indicate module hubs (see Figure 5). S, seedling; F, flowering; H, harvest.

These key OTUs belonged to genera such as *Rhizobium*, *Sphingomonas*, *Burkholderia* and *Pseudomonas*, which are known to contain strains with plant growth-promoting abilities (Mendes et al., 2013). But also, less characterised genera such as *Massilia*. The genus *Massilia* has been found to be abundant in the rhizosphere of many plant species (Bodenhausen et al., 2013; Yeoh et al., 2016) and to be associated with soils colonised by pathogenic fungi (Yin et al., 2013). As several *Massilia* isolates are able to degrade chitin (Adrangi et al., 2010) found in fungal cell wall, it is possible that these strains may be acting as fungal antagonists. The ability to produce extracellular chitinases is considered crucial for *Serratia marcescens* to act as antagonist against *Sclerotium rolfsii* (Chet et al., 1990).

We constructed microbial networks for each of the six temporal sampling points (Supplementary data Figure S13, Supplementary data Table S5) in order to identify OTUs that may be involved in the maintenance of community structure and function (Power et al., 1996). Using random matrix theory (Deng et al., 2012), we could not identify any OTU playing a significant role in maintaining microbial community structure in bulk soil samples. However, we found 47 OTUs (representing 21.2% of the total number of reads) that may be important in structuring rhizosphere microbial communities (Figure 5, Supplementary data Table S5). Rhizosphere networks have been shown to be substantially more complex than those of bulk soils (Shi et al., 2016). These patterns likely occur because microbial species share niche space in the rhizosphere and, as a result, show strong positive associations in networks (Mendes et al., 2014; Edwards et al., 2015).

The sorghum and sunflower rhizosphere networks contained taxa with module hubs properties (high *Zi*, low *Pi*; 25 OTUs), i.e., highly connected OTUs linked to many OTUs within their own module. Taxa classified as connectors (low *Zi*, high *Pi*; 22 OTUs), which link several modules,

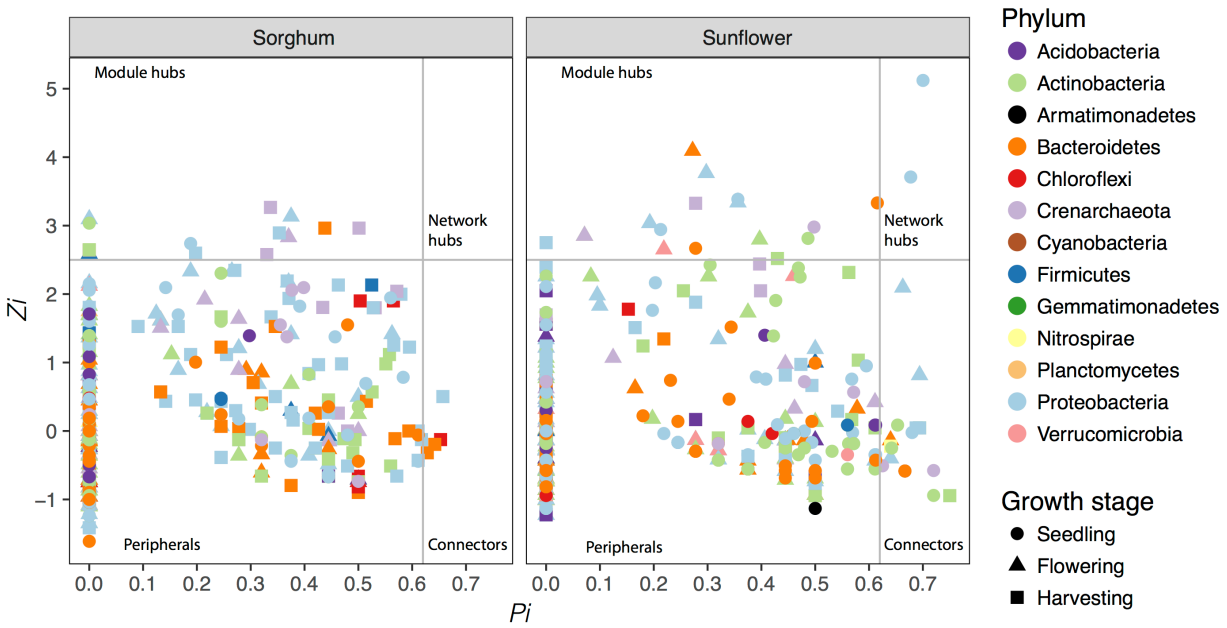


Figure 5. Classification of nodes (OTUs) to identify putative keystone species within the rhizosphere networks. Modules hubs (highly connected OTUs within modules) have Zi > 2.5, connectors (OTUs that connect modules) have Pi > 0.62 and network hubs (highly connected OTUs within the entire network) have Zi > 2.5 and Pi > 0.62 (Guimerà and Amaral, 2005).

and network hubs (with both a high *Zi* and a high *Pi*; 2 OTUs) (Supplementary data Table S5) were also present. Module hubs were detected in all six networks, most belonging to the *Proteobacteria* (12 OTUs; 7 from *Alphaproteobacteria*, 4 from *Gammaproteobacteria* and 1 from *Betaproteobacteria*), *Crenarchaeota* (7 OTUs) and *Actinobacteria* (6 OTUs). In contrast, connectors were detected in only four of the six rhizospheric networks (Figure 5, Supplementary data Table S5 and Figure S13). Seven of these connectors were *Proteobacteria* (all *Alphaproteobacteria*), 5 *Actinobacteria* and 5 *Bacteroidetes*. The two network hubs were found in the sunflower rhizosphere at seedling (Supplementary data Table S5) and belonged to the *Alphaproteobacteria*.

Taxa identified as network and module hubs, and connectors, are thought to be keystone taxa due to their role in maintaining network structures (Deng et al., 2012; Olesen et al., 2007). The disappearance of these putative keystone taxa may cause modules and networks to disassemble (Power et al., 1996), and thus keystone taxa may play a role in maintaining ecosystem stability (Olesen et al., 2007). Based on this criterion, members of the phylum *Proteobacteria* are the most prominent keystone taxa in the sunflower and sorghum rhizosphere networks, as they accounted for 42% of all network hubs, module hubs and connectors. Interestingly, five of the module hubs (with one OTU related to each of the genera *Rhizoplanes*, *Flavisolibacter*, *Povalibacter* and two OTUs belonging to *Nitrososphaera*) were structurally important in at least two rhizosphere networks (Supplementary data Table S5) and two, classified as *Rhizoplanes* and *Lysobacter*, were more abundant in rhizosphere than in bulk soil at some growth stage (Figure 4). In addition, a module hub (genus *Sphingomonas*) in the sorghum rhizosphere at harvest also showed network hub properties in the sunflower rhizosphere at seedling stage (Supplementary data Table S5). A second module hub (genus *Nitrososphaera*) in the sorghum rhizosphere at

flowering also showed connector properties in the sunflower rhizosphere at planting. Overall, these results suggest that the same taxon may play an identical structural role at different developmental stages and in different crops. This is in contrast to a previous study that found that the putative keystone taxa in the rhizosphere of *Avena fatua* changed over time (Shi et al., 2016); i.e., no single taxon acted as hub or connector in two different networks. Knowledge of the specific ecological role of most of these bacteria in soils is limited, although several of the above mentioned taxa, which have been isolated from a variety of plant samples, may provide beneficial effects on plant health and growth (Mendes et al., 2013). For instance, a number of *Lysobacter* species are known to have strong antagonistic activities against a range of pathogens (Expósito et al., 2015).

4. CONCLUSION

Here we assessed spatial and temporal changes of microbial (bacterial and archaeal) communities in the rhizosphere of sorghum and sunflower grown in crop rotation. We show that rhizospheric soils of both sorghum and sunflower contain taxa commonly found in the rhizosphere of other plant species, together with taxa previously reported as only minor components of rhizosphere communities (e.g., archaea). The relative abundance of most microbial phylotypes (phylum and family levels) remained relatively stable through the sampling period and with no differences between the rhizosphere and the bulk soil, probably because bulk soils contained root debris from the same crops planted in the previous two years. However, some phyla and families were enriched in the rhizosphere relative to the bulk soil at all stages. In contrast, a larger number of individual taxa (OTUs delimited at 97% similarity level) were differentially more abundant in one of the two habitats. Interestingly, some of these taxa show

similar abundance and structural patterns at different sampling times, suggesting that the same organisms may play the same functional/structural role at different plant growth stages and in different crops. Although to infer function from sequencing data is problematic, we noted that sorghum and sunflower rhizospheres shared key taxa belonging to genera such as *Sphingomonas* and *Rhizobium*, which are known to containing strains that promote plant health and growth, as well as taxa potentially important in maintaining the structure of microbial communities. Further investigations using targeted isolation and further characterization will provide a better understanding of the role of these microorganisms in the rhizosphere environment. Characterization efforts could make use of recent advances in microfluidic technology (Stanley and van der Heijden, 2017) and system biology approaches (Agler et al., 2016) to identify efficient microbial consortia that can be used as bioinoculants to optimise crop growth.

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Availability of Data and Materials

The raw sequencing reads and metadata for this project were submitted to the National Centre for Biotechnology Information Short Read Archive under accession no. SRP110648.

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Supplementary material

Supplementary data Table S1. Good's coverage estimates indicating percentage of OTUs sampled.

Supplementary data Table S2. Soil chemistry. All concentrations are expressed in mg/kg.

Supplementary data Table S3. OTUs (137 in total) showing relative abundances higher than 0.1%. These OTUs represented 43.3% of the total number of reads and belonged to the bacterial phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and the archaeal phylum *Crenarchaeota*.

Supplementary data Table S4. Differentially abundant OTUs (Bulk soil *vs* Rhizosphere soil).

Supplementary data Table S5. Key rhizosphere OTUs. Defined as those found in greater abundance in the rhizosphere compared to the bulk soil in at least two growth stages and/or being module hubs, networks hubs and connectors in rhizosphere networks.

Supplementary data Figure S1. a) Average Good's coverage estimates (%) and b) rarefaction curves. BS, bulk soil; RS, rhizosphere soil. S, seedling; F, flowering; H, harvest. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old

Supplementary data Figure S2. PCoA of soil parameters at pre-planting, using Euclidean distances with standardized data, and PERMANOVA tables. P-values were obtained after

correction for multiple comparisons using Benjamini-Hochberg discovery rate. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old.

Supplementary data Figure S3. Faith's phylogenetic diversity (PD) at pre-planting. Different letters indicate significant differences in PD (Wilcoxon-Mann-Whitney $P < 0.05$) between soils. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old.

Supplementary data Figure S4. PCoA of soil bacterial communities at pre-planting, using weighted UniFrac distances, and PERMANOVA tables. P-values were obtained after correction for multiple comparisons using Benjamini-Hochberg discovery rate. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old.

Supplementary data Figure S5. Faith's phylogenetic diversity (PD), richness and Chao1 estimator. Least-square mean for each crop (So, sorghum; Su, sunflower) is plotted with ± 1 s.e of the mean. Different letters indicate significant differences (ANOVA $P < 0.05$).

Supplementary data Figure S6. PCoA of post-planting soil bacterial communities, using weighted UniFrac distances, and PERMANOVA tables. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old. So, sorghum; Su, sunflower.

Supplementary data Figure S7. PCoA of post-planting soil parameters, using Euclidean distances with standardized data, and PERMANOVA tables. LB, Limpopo black; LR, Limpopo red; FN, Free State new; FO, Free State old. So, sorghum; Su, sunflower.

Supplementary data Figure S8. Soil chemistry. All concentrations are expressed in mg/kg

Supplementary data Figure S9. Relative abundance over time of the nine most abundant phyla. PP, pre-planting; S, seedling; F, flowering; H, harvest.

Supplementary data Figure S10. Abundance of the phyla enriched in each habitat type (bulk and rhizosphere soils). S, seedling; F, flowering; H, harvest.

Supplementary data Figure S11. Relative abundance over time of the ten most abundant families. PP, pre-planting; S, seedling; F, flowering; H, harvest.

Supplementary data Figure S12. Abundance of the families enriched in each habitat type (bulk and rhizosphere soils).

Supplementary data Figure S13. Rhizosphere networks a) sorghum at seedling, b) sorghum at flowering, c) sorghum at harvest, d) sunflower at seedling, e) sunflower at flowering, f) sunflower at harvest. C, connectors; MH, module hubs; NH, network hubs. Bacteria are depicted as ellipses and archaea as squares.