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ORIGINAL PAPER

Microbial 16S gene-based composition of a sorghum cropped rhizosphere soil under different fertilization managements

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Abstract Driven by growing desertification and increases in the global demand for food, the necessity to adopt sustainable fertilization and crop management systems have greatly increased. In dryland soils, certain crops such as sorghum could provide important advantages. The impact of 3 years of N-fertilization with two rates of compost amendment on the composition of bacterial community of a Mediterranean soil cropped to sorghum was evaluated. The composition of bacterial communities of rhizospheric soil samples fertilized by urea (CT) or compost at single (COM1) or double doses (COM2), were compared to that of the bacterial communities from unfertilized rhizospheric soil (UF) and grassland soil (GS) by pyrosequencing. The highest number of sequences and OTUs were associated with rhizosphere soils treated with the double dose compost amendment (COM2), and analysis of alpha diversity clearly indicated a higher richness of this treated soil than other soils. Of the 16 bacterial phyla observed, *Actinobacteria* and *Proteobacteria* dominated. *Actinobacteria* abundance was higher in both compost-amended soils (COM1 and COM2) and GS than other investigated soils; *Proteobacteria* had the opposite trend. Significant differences ($P<0.05$) were detected among class compositions of treatments. Most of the screened families belonged to α-*Proteobacteria* class. Species level analysis showed that GS and COM2-treated soil presented the highest percentage of unique OTUs; for 8 of the 14 most abundant OTUs, significant differences ($P<0.05$) were found among soils. A clear distinction of bacterial communities of soil under different fertilization managements was observed from weighted as well as unweighted PCoA plots. Results from this in depth analysis clearly indicated that organic fertilization by compost, more than chemical fertilization by urea, can affect the composition of bacterial communities inhabiting the sorghum rhizosphere when compared to unfertilized soil and grassland soil.

Keywords Urea fertilization · Compost amendment · Sorghum crop · Rhizosphere · Soil bacterial community · Pyrosequencing

Introduction

Soil microorganisms play important roles in soil processes; they regulate C and N cycling, provide nutrients to plants, and play pivotal roles in soil structure and fertility. Many biotic and abiotic factors may alter the microbial community, which may in turn directly or indirectly influence the soil ecosystem.

Chemical and organic fertilizers and plant rhizodeposition can influence the composition and function of soil bacterial communities (Marschner et al. 2001; Bulluck et al. 2002; Pérez-Piqueres et al. 2006; Zhang et al. 2008; Acosta-Martínez et al. 2010; Wang et al. 2012). For instance, urea is a widely used chemical fertilizer that promotes plant growth (Taiz and Zeiger 2010; Bashir et al. 2012) and increases soil organic matter content (Stevenson 1986) but may have both positive and negative effects on activity of soil bacterial communities (Lee and Caporn 1998; Sarathchandra et al. 2001).

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These contrasting effects are probably due to differences in the amounts of N applied in different experiments as well as to the specific ecosystem, seasons, and plants (Zhang et al. 2008).

Although chemical fertilizers are still widely used, organic amendments are being rapidly adopted. Studies on soil organic amendment, performed both in microcosms and in field experiments, show that compost amendment not only improves soil structure but, by enhancing soil organic matter (SOM) content, also strongly influences activity, biomass, and composition of soil microflora (Crecchio et al. 2001). As both N fertilization and compost amendment improve a soil organic matter content, soils, such as those in Mediterranean area characterized by low levels of SOM, may benefit from both management practices.

Specialized crops adapted for growth in particular climates could similarly represent another important step toward sustainable agriculture. To this purpose, sorghum has attracted increasing interest in Mediterranean regions (Coelho et al. 2007). Due to its tolerance of harsh environments, sorghum could be a valuable alternative to the less drought-resistant and largely utilized wheat. However, despite the large number of studies about the effects of wheat and other cereals on the composition of soil microbial communities (Lupwayi et al. 1998; Drijber et al. 2000; Smalla et al. 2001; Mäder et al. 2002; Zhao et al. 2014), as highlighted by Acosta-Martínez et al. (2010), the literature on the influence of sorghum on composition of soil microbial communities is very limited.

In this context, this work evaluated the impact of 3 years of N-fertilization and compost amendment on the composition of bacterial communities of a Mediterranean soil cropped to sorghum. Molecular approaches such as the 16S rRNA gene-based high throughput pyrosequencing are widely used to investigate responses of the composition of soil microbial communities to different factors (Lauber et al. 2009; Rousk et al. 2010; Nacke et al. 2011; Yang et al. 2012; Lagos et al. 2014). To our knowledge, only the studies of Acosta-Martínez et al. (2010) and Ng et al. (2012) evaluated composition of microbial communities in dryland cropping systems under different tillage practices by this approach, and the composition of sorghum rhizospheric microbial communities under different fertilization managements have not yet been examined by high throughput sequencing. Thus, this study was performed to determine the responses of the composition of microbial communities in sorghum-cropped soil to different levels of organic and chemical fertilization.

Materials and methods

Site description, experimental design, and sampling

The experimental site was established on the CRA-ORT (Centro di Ricerca per l'Orticoltura) agricultural farm near Battipaglia (SA), Italy, (40° 61′ N and 14° 98′ E). The soil was classified as Pachic Phaeozems and characterized by low organic C (OC) content (11.39 g kg^{-1}). The five managements encompassed four traditionally plowed and irrigated sorghum cropped plots under different N fertilizer amendments and an unmanaged grassland soil covered by spontaneous vegetation (GS). The four managed soils were as follows: chemically fertilized by 130 kg of urea-N ha^{-1} (CT), compost-amended by 130 kg of N ha^{-1} (COM1), compost-amended by 260 kg of N ha^{-1} (COM2), and unfertilized (UF). The green compost was produced on the farm with 60 days maturity and C/N average ratio of 25. The experimental design utilized 5 m× 8 m plots and was a completely randomized block with four replicates (a, b, c, d) for each soil treatment.

After 3 years of management, rhizospheric soil samples were collected for each cropped plot. Rhizospheric soil was collected during feekes stage 4–5 by pulling three plants per plot. Plants were first shaken gently to separate the root system from the bulk soil. Then, soil tightly adhering to the roots was scrubbed in sterile condition. A composite grassland soil was obtained by removing the litter layer and by sampling five cores at a random location within the plot; the soil subsamples were pooled, passed through a 2 mm sieve, and immediately stored at 4 °C.

DNA extraction, 16S rDNA amplification, and pyrosequencing

Total microbial community DNA was extracted from approximately 0.5 g of each soil sample using the Thermo Savant FastPrep® System homogenizer combined with the FastDNA®SPIN Kit for Soil (MP Biomedicals, LLC) according to the manufacturer's protocol. The quality of DNA extracts was evaluated by 0.7 % agarose gels in 0.5 X Tris-Borate–EDTA buffer, and its concentration was determined by spectrophotometric measurements at 230, 260, and 280 nm using a NanoDrop® ND-1000 UV–vis spectrophotometer (ThermoFisher Scientific Inc.).

The V1–V3 region of the 16S rRNA gene was PCR amplified as described by Garcia et al. (2011) using 27F-YM+3, a sevenfold-degenerate primer (Frank et al. 2008), and 515R-NK (5′-CCGCNGCKGCTGGCAC-3′), which was modified after Acosta-Martínez et al. (2008). The primers contained the Roche 454-pyrosequencing adaptors B and A, respectively. In addition, the 515R-NK primer contained an 8-nt-long sample specific barcode for post-sequencing bioinformatics separation of sequences of each sample. PCR conditions, thermal parameters, and processing of amplicons for pyrosequencing were those according to Garcia et al. (2011). The sequencing was performed at the Georgia Genomics Facility at the University of Georgia (Athens, USA) on a Roche GS-FLX 454 pyrosequencer (Roche Diagnostics Corporation). The

sequences were submitted to SRA with accession number SRP055695.

Alpha and beta diversity estimation, taxonomy, and statistical analysis

The pyrosequencing-derived dataset were computationally screened by two different open-source packages as described by Garcia et al. (2011). Briefly, denoising and separation of sequences to sample libraries was done using QIIME v1.2.0 (Quantitative Insights Into Microbial Ecology) (http://qiime.sourceforge.net/) (Caporaso et al. 2010). Then, MOTHUR v1.12.0 (http://www.mothur.org/) was used to perform sequence alignment by SILVA aligner database and all other sequence processing (Schloss et al. 2009). Based on the alignment, a distance matrix was constructed, and pair-wise distances used for clustering the sequences into Operational Taxonomic Units (OTUs) at a genetic distance of 0.03 (D=0.03).

Clustering at 3 % was used to evaluate general diversity by rarefaction curves and other measures. The Shannon diversity index (H) was used as a measure of general diversity and evenness. The reciprocal of Simpson's index (1/D) was also calculated. ACE and Chao1 were computationally calculated as richness estimators. The taxonomic affiliation of each OTU was calculated by the MOTHUR command classify.seqs using the SILVA bacterial taxonomy database. PermutMatrix software (Caraux and Pinloche 2005) was used to generate a heat map of bacterial diversity by hierarchical clustering using the Manhattan distance method with no scaling and unweighted-pair technique.

The shared file output from MOTHUR was used to generate an OTUs network using Cytoscape v3.0.2 and to analyze shared and most abundant OTUs. Evaluation of community similarity between samples according to their composition and membership was assessed by using weighted and unweighted UniFrac distance matrix (Lozupone and Knight 2005) for principal coordinate analysis (PCoA). All statistical differences were performed by ANOVA, and means were segregated by the SNK multiple comparison test at $P \leq 0.05$, by using SPSS package (SPSS Inc., v.21, Chicago, Illinois).

Results

Alpha diversity

The computational screening of the pyrosequencing output from the 20 samples (5 treatments × 4 replicates) generated 90,406 sequences (Table 1). More than 50 % of these sequences were associated with the double dose compost amendment (COM2). About 17 % of the sequences were associated to the single dose compost amendment (COM1), and 13 and 10 % were from the grassland (GS) and unfertilized soils (UF), respectively. The lowest percentage was from the chemically fertilized plots (CT). Sequence clustering to 3 % dissimilarity produced 42,412 OTUs for the entire study site.

The rarefaction curves clearly indicate the absence of saturation and a higher richness for COM2 than for other agronomic managements (Fig. 1). Because of the wide range of sample sizes among treatments, the number of sequences was standardized for all samples in order to obtain unbiased estimates of the diversity, as suggested by Schloss et al. (2011). The minimum number of sequences, corresponding to the smallest replicate of sample CT was 1161 sequences. Therefore, this number of sequences was randomly selected from each replicate to yield 4644 sequences per sample in the normalized dataset. The reduced dataset was then used to calculate the community richness and all further OTU and sequence elaborations (Table 1). The community richness, evaluated by rarefaction curves on the normalized dataset (Fig. 1, inset), still demonstrated a lack of saturation as well as higher richness of soil microbial communities of the COM2 amended soil. Diversity was further evaluated at 3 % dissimilarity with the Shannon (H) and Simpson's (1/D) diversity indices, non-parametric estimators of richness Chao1 and ACE, and Shannon evenness (H/log(S)) (Table 1). The SNK-test applied to Shannon indices of diversity and evenness failed to detect significant differences among the management treatments. In contrast, the reciprocal of Simpson's index found significant differences between the GS and COM1 plots, with the other plots being of intermediate diversity but not significantly different. A significantly higher richness (P<0.05) was found for soils amended with the double dose of compost (COM2) than for other soil samples, as indicated by the non-parametric estimators of richness CHAO 1 and ACE (Table 1) and by the rarefaction curves.

Composition of bacterial communities

The alignment of normalized sequences dataset with the SILVA database identified 16 bacterial phyla (Table 2). *Actinobacteria* and *Proteobacteria* were the most abundant phyla, amounting to 40.3 and 35.1 % of the total number of sequences, respectively. Less abundant phyla were *Bacteroidetes* (3.0 %), *Acidobacteria* (2.9 %), *Gemmatimonadetes* (2.4 %), *Firmicutes* (1.7 %), and *Chloroflexi* (1.4 %). *Candidate_division_TM7*, *Candidate_division_OP10*, *Chlamydiae*, *Cyanobacteria*, *Deinococcus-Thermus*, *Nitrospirae*, *Planctomycetes*, and *Verrucomicrobia* were all represented by less than 1 % of the total number of sequences. The remaining 10.9 % of total sequences consisted of unclassified bacteria. All identified phyla were common to the differently managed soils except for *Chlamydiae* and *Deinococcus-Thermus*, which were only found in GS and CT, respectively.

Table 1 Diversity indices of soil microbial communities under different managements

Diversity index	Cropland				Grassland
	Unfertilized	Chemical fertilized	Compost amended		
	UF	CT	COM1	COM2	GS
Number of sequences[a]	8541 (4644)	6870 (4644)	15,084 (4644)	48,286 (4644)	11,625 (4644)
OTUs (S)	3919 (2924)	3069 (2811)	5569 (2764)	23286 (3408)	6569 (3335)
Shannon index (*H*)	6.61	6.61	6.60	6.77	6.78
Eveness=*H*/log (*S*)	1.90	1.92	1.91	1.92	1.92
Reciprocal of Simpson's index(1/D)	972 ab	1084 ab	481 a	1090 ab	1255 b
Chao 1	4329 a	3350 a	3637 a	8371 b	6095 ab
ACE	6410 a	6785 a	7058 a	22041 b	7263 a

Calculations were based on OTUs at an evolutionary distance of 0.03 (97 % similarity). All measures are means of four replicates of each sample; data with different letters in each row are significantly different, according to SNK-test at $P<0.05$

[a] Values correspond to the complete dataset of sequences while values in brackets refer to a subset of sequences (see text for explanation)

The distribution of each phylum among the differently treated plots was evaluated by SNK-tests. *Actinobacteria* abundance was lower in CT and UF than in both compost amended soils and GS. *Proteobacteria* abundance showed an opposite trend; its abundance ranked as CT-UF>COM2>COM1-GS soils. *Bacteroidetes* were more abundant on all managed soils than GS plots. Conversely, grassland soil was characterized by a higher abundance of *Acidobacteria*. The abundances of many of the other phyla were not significantly different between the plots. Some exceptions included the *Gemmatimonadetes*, which were significantly more abundant in the UF plots than the COM1 and CT plots; the *Firmicutes*, which were significantly more abundant in the CT than the COM1 plot; the *Chloroflexi*, which was significantly more abundant in the UF than the CT plot; and *Candidate_division_TM7*, which was significantly more abundant in the COM1 than all other plots and significantly less abundant in the GS than the remaining plots. The distribution of all other phyla, each representing less than 1 % of the total number of sequences, were generally not significantly different among different managements. The distribution of the unclassified bacteria was also very similar for all the managed soils but somewhat higher in GS plots.

Evidence for further differences in the composition of bacterial communities at the taxonomic level of class was also found (Table 3). Classes which comprised less than 1 % of the sequences were not considered further. The most abundant classes were the *Actinobacteria* and *α-Proteobacteria*, comprising 69 % of the total number of sequences. Less abundant classes included the *β-Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Gemmatimonadetes*, *Bacilli*, and *γ- and δ-Proteobacteria*. Ten percent of total sequences were

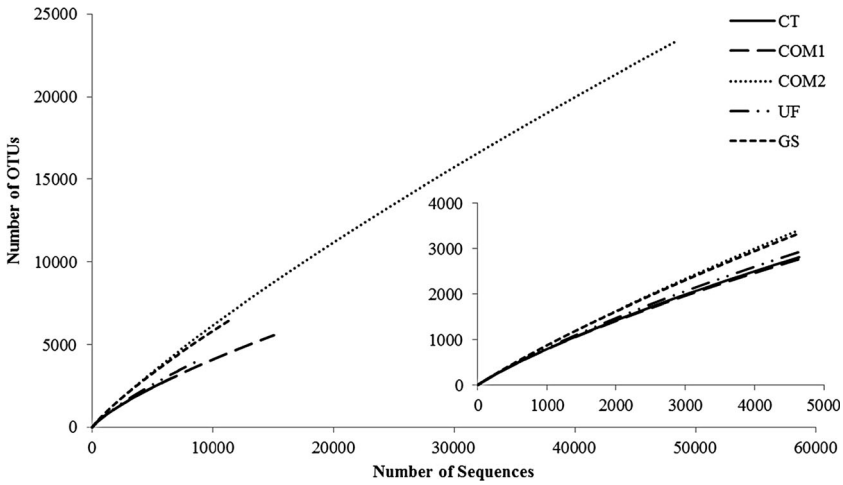

Fig. 1 Rarefaction curves of the complete dataset of sequences (main plot) and the randomly chosen subset of 4644 sequences. OTUs were defined at a genetic distance of 0.03. *CT* chemical fertilization, *UF* not fertilized, *COM2* double dose compost amendment, *COM1* single dose compost amendment, *GS* grassland soil

Table 2 Phylotype abundance in the different soil libraries

Phyla	Cropland				Grassland	All sites
	Unfertilized	Chemical fertilized	Compost amended			
	UF	CT	COM1	COM2	GS	
Acidobacteria	113 a	117 a	131 a	104 a	212 b	677
Actinobacteria	1746 a	1690 a	2017 b	1898 b	2010 b	9361
Bacteroidetes	130 b	160 b	165 b	177 b	65 a	697
Candidate_division_TM7	32 b	20 ab	111 c	34 b	8 a	205
Candidate_division_OP10	2	6	3	3	2	16
Chlamydiae	0	0	0	0	1	1
Chloroflexi	81 b	46 a	69 ab	59 ab	68 ab	323
Cyanobacteria	8	6	6	9	4	33
Deinococcus-Thermus	0	2	0	0	0	2
Firmicutes	77 ab	103 b	60 a	78 ab	78 ab	396
Gemmatimonadetes	141 b	118 ab	118 ab	80 a	89 a	546
Nitrospirae	11 a	17 ab	12 ab	8 a	21 b	72
Planctomycetes	11	17	7	11	21	67
Proteobacteria	1810 c	1858 c	1469 a	1658 b	1347 a	8142
Verrucomicrobia	32	32	27	17	33	141
Unclassified bacteria	454 a	452 a	449 a	508 a	678 b	2541
Sample size	4644	4644	4644	4644	4644	23220

Total number of sequences is presented after normalizing for sample size; data with different letters in each row are significantly different, according to SNK-test at $P<0.05$

associated to unclassified bacteria. By comparing class composition among treatments, significant differences ($P<0.05$) were detected for all classes accounting for more than 1 % of the total number of sequences (Table 3). In particular, *Acidobacteria*, *Actinobacteria*, *Sphingobacteria*, *Gemmatimonadetes*, and *Bacilli* classes showed the same trend as their phyla. In contrast, the δ and γ-*Proteobacteria* classes were more abundant in GS and UF soil than in fertilized soils, respectively (Table 3).

The distribution of the most abundant families in the *Actinobacteria* and α-*Proteobacteria* classes was also examined (Fig. 2). Twenty-five families were found, most of them belonged to α-*Proteobacteria*, *and* only five belonged to the *Actinobacteria* class. The families were distributed into two major clusters (A and B) and four sub-clusters (A1 and A2; B1 and B2). The A cluster families were of low abundance in the GS plots and distinguished largely by differences in their abundances in the treated plots. Subcluster A1 was abundant in UF soil as well as COM2 and CT samples. Members of the *Bradyrhizobiaceae* were only present in the UF soils. Within the same sub-cluster, large differences in abundances were found for the COM1 and COM2 amended soils, indicative of major differences between the communities in these two rhizospheric soils. In constrast, COM1 and COM2 had very similar compositions for families in subcluster A2, suggesting a certain amount of functional parity for these taxa. The B1 subcluster was abundant in the GS soil but not the other soils. Especially dramatic were the abundances of members of the families of *Methylocystaceae*, *Actinobacteridae_uncl.*, and *Hyphomonadaceae*, which were not detected in the other samples. Lastly, the B2 subcluster was only abundant in the CT soil and included *Rickettsiae* and *Methylobacteriaceae*.

Finally, soil bacterial diversity was investigated to the species level (OTUs at $D=0.03$). The OTUs list of the shared file output from MOTHUR was used to generate an OTU network showing unique and shared OTUs (Fig. 3) and to analyze the most abundant OTUs (Table 4). The total number of OTUs found for the entire subsample was 11,913. GS and COM2 soils had the highest percentages of unique OTUs, about 22 and 20 %, respectively (Fig. 3). CT and UF were characterized by a comparable percentage of unique OTUs, about 14 %, while COM1 soil had the fewest percentage (13 %) of unique OTUs. About 16 % of the total number of OTUs was shared among all samples.

Distribution of the most abundant OTUs was also evaluated (Table 4). Significant differences ($P<0.05$) in the distributions were found for 8 of the 14 most abundant OTUs. Of these, OTU003_*Alphaproteobacteria* and OTU0014_*Actinobacteria* were much more abundant in the CT and GS soils, respectively, than the other soils; in contrast, OTU001_*Actinobacteria* and OTU004_*Alphaproteobacteria* were most abundant in the compost amended soils.

Table 3 Relative abundance of phylogenetic classes in the different fertilization managements

Phyla	Class[a]	Cropland				Grassland	All sites
		Unfertilized	Chemical fertilized	Compost amended			
		UF	CT	COM1	COM2	GS	
Acidobacteria	*Acidobacteria**	2.39 b	2.43 b	2.82 b	2.15 b	4.37 a	2.8
	Unclassified	0.04	0.09	0.00	0.09	0.19	0.1
Actinobacteria	*Actinobacteria**	37.60 b	36.39 b	43.43 a	40.87 a	43.28 a	40.3
Bacteroidetes	*Flavobacteria*	0.26 ab	0.54 a	0.13 b	0.13 b	0.02 b	0.2
	*Sphingobacteria**	2.50 a	2.69 a	3.21 a	3.47 a	1.29 b	2.6
	Unclassified	0.04	0.22	0.22	0.22	0.09	0.2
Candidate_division_TM7	*Unclassified*	0.69 b	0.43 ab	2.39 a	0.73 b	0.17 c	0.9
Candidate_division_OP10	*Unclassified*	0.04	0.13	0.06	0.06	0.04	0.1
Chlamydiae	*Chlamydiae*	0.00	0.00	0.00	0.00	0.02	0.0
Chloroflexi	*Anaerolineae*	1.29 a	0.60 b	0.86 ab	0.69 ab	1.08 ab	0.9
	Thermomicrobia	0.30	0.28	0.58	0.52	0.30	0.4
	Unclassified	0.15	0.11	0.04	0.06	0.09	0.1
Cyanobacteria	*Chloroplast*	0.00	0.00	0.00	0.00	0.02	0.0
	SubsectionI	0.02	0.04	0.02	0.02	0.02	0.0
	SubsectionIV	0.00	0.00	0.00	0.00	0.04	0.0
	Unclassified	0.15	0.09	0.11	0.17	0.00	0.1
Deinococcus-Thermus	*Deinococci*	0.00	0.04	0.00	0.00	0.00	0.0
Firmicutes	*Bacilli**	1.64 ab	2.02 a	1.18 b	1.61 ab	1.51 ab	1.6
	Clostridia	0.02	0.17	0.09	0.04	0.09	0.1
	Erysipelotrichi	0.00	0.00	0.00	0.00	0.02	0.0
	Unclassified	0.00	0.02	0.02	0.02	0.06	0.0
Gemmatimonadetes	*Gemmatimonadetes**	3.04 a	2.54 ab	2.54 ab	1.72 b	1.92 b	2.4
Nitrospirae	*Nitrospira*	0.15 b	0.37 ab	0.26 ab	0.17 b	0.60 a	0.3
Planctomycetes	*Phycisphaerae*	0.11	0.09	0.09	0.04	0.04	0.1
	Planctomycetacia	0.13	0.26	0.06	0.13	0.34	0.2
	Unclassified	0.00	0.02	0.00	0.06	0.06	0.0
Proteobacteria	*Alphaproteobacteria**	31.59 a	32.15 a	26.44 b	30.00 a	23.34 c	28.7
	*Betaproteobacteria**	3.96 ab	4.09 a	2.78 cd	2.91 bc	1.92 d	3.1
	*Deltaproteobacteria**	0.69b	0.93 b	1.01 b	0.47 b	1.89 a	1.0
	*Gammaproteobacteria**	2.22 a	2.05 a	1.14 b	1.83 ab	1.10 b	1.7
	Unclassified	0.52 ab	0.80 a	0.26 b	0.50 ab	0.75 a	0.6
Verrucomicrobia	*OPB35*	0.13	0.11	0.19	0.06	0.17	0.1
	Opitutae	0.06	0.19	0.09	0.06	0.06	0.1
	Spartobacteria	0.17	0.09	0.15	0.15	0.32	0.2
	Verrucomicrobiae	0.15	0.13	0.13	0.02	0.02	0.1
	Unclassified	0.17	0.17	0.02	0.06	0.13	0.1
Unclassified bacteria	*Unclassified*	9.78 b	9.73 b	9.67 b	10.94 b	14.60 a	10.9

Values reported are the % of each library. Values with different letters in each row are significantly different according to SNK-test at $P<0.05$

[a] Classes indicated by asterisk contain more than 1 % of the total number of sequences

Beta diversity

Other analyses demonstrated clear distinctions of the bacterial communities under different fertilization managements in both community composition (weighted/quantitative) and membership (unweighted/qualitative), assessed by UniFrac-PCoA (Fig. 4). Axis 1, which explains the majority of variations in the data, clearly separates all fertilized and unfertilized sorghum rhizospheric soils from the grassland soil. Axis 2 possessed distinct results when weighted and unweighted

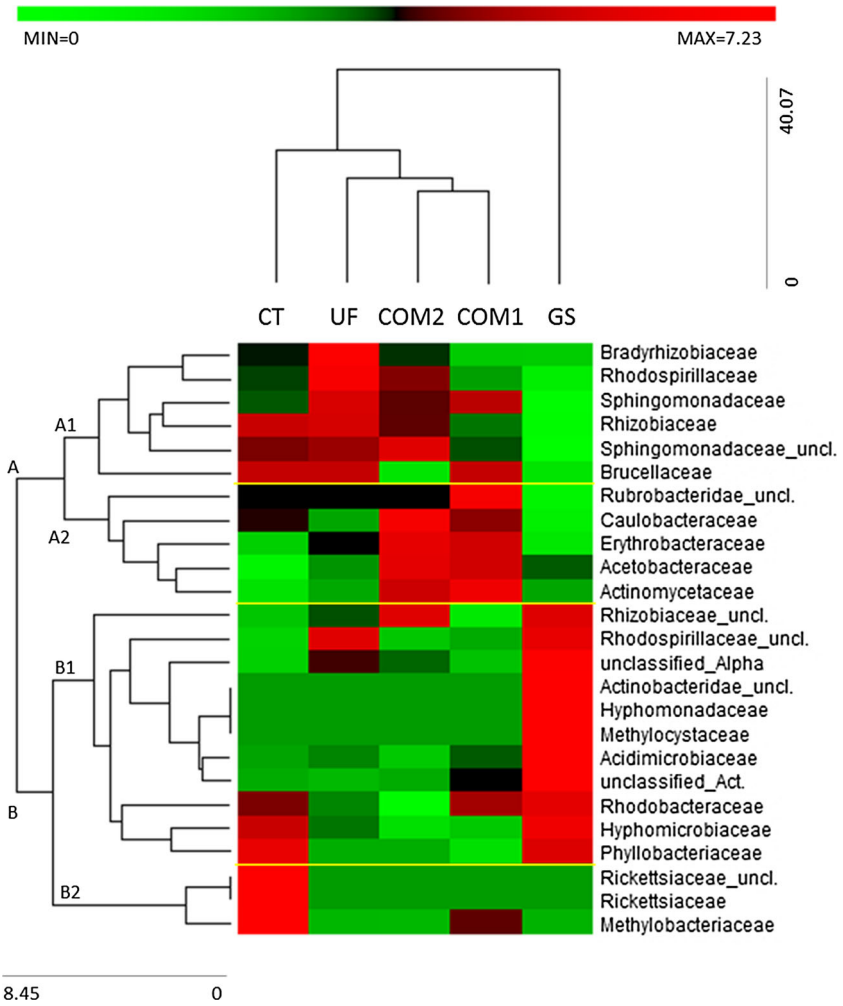

Fig. 2 Heat map depicting bacterial diversity across the different soil managements. The hierarchical dendrogram shows the composition and distribution of families and classes (for *Actinobacteria* and *α-Proteobacteria*) across soil samples. Clustering in the X-direction indicates divergence in phylogenetic composition. Clustering in the Y-direction indicates divergence in abundance not phylogenetic similarity. The scale at the top defines the relative abundance of the phylogenetic groups as depicted by the colors in the heat map. *CT* chemical fertilization, *UF* not fertilized, *COM2* double dose compost amendment, *COM1* single dose compost amendment, *GS* grassland soil

PCoA were considered. In the PCoA of the weighted UniFrac distance matrix, only COM2 rhizosphere soil was clearly separated from the other managed soils, while the PCoA of the unweighted UniFrac also separated COM1 rhizosphere soil.

Discussion

Like most investigations (Nacke et al. 2011; Chaudhry et al. 2012), our results on alpha diversity indicated that, despite the quite high number of sequences obtained by the pyrosequencing of the 16S rDNA amplicons, the whole communities were not completely covered (since the saturation in the rarefaction curves was not got). However, as already reported by Jangid et al. (2008) and Shange et al. (2012), this does not constitute a limit for the investigation on bacterial diversity. Both the number of sequences and OTUs were significantly higher in compost-amended soils than in other soils with the highest richness of bacterial community in the double compost dose.

Actinobacteria were the dominant soil bacterial taxa confirming what found by Gremion et al. (2003) and Janssen (2006). The role of this taxa in the decomposition of organic materials is well established (Lacey 1973). We found a high abundance of *Actinobacteria* in compost-amended soils, in agreement with Acosta-Martínez et al. (2008), as well as in grassland soil; this finding confirms, as hypothesized by Kopecky et al. (2011), that *Actinobacteria* can be expected in soil ecosystems with different quantities and quality of organic matter. However, it is interested that the A*ctinobacteria* species abundant in the compost-amended soils were not always abundant in the grassland soil, indicating the great diversity of this phylum. Similarly, we found a low abundance of *Actinobacteria* in unfertilized soil and, in agreement with Koyama et al. (2014), in soil treated with chemical fertilizers. In contrast, Chaudhry et al. (2012) and Ai et al. (2015) found that *Actinobacteria* were more abundant in chemically fertilized soil than in organic managed soil. Many factors such as temperature, pH, and vegetation could be responsible of the different responses of *Actinobacteria* abundance and diversity (Youssef and Elshahed 2009). Thus, it is difficult to correlate the presence and the functions of these bacteria to particular soil conditions (Hackl et al. 2004; Bachar et al. 2010).

Table 4 Most abundant OTUs in the 16S rRNA gene libraries

OTUs_class affiliation[a]	Cropland				Grassland	All sites[b]
	Unfertilized	Chemical fertilized	Compost amended			
	UF	CT	COM1	COM2	GS	
OTU001_*Actinobacteria*	36 b	14 c	81 a	50 ab	8 c	189
OTU002_*Alphaproteobacteria*	56 a	44 a	36 a	35 a	0 b	171
OTU003_*Alphaproteobacteria*	0 b	146 a	0 b	0 b	1 b	147
OTU004_*Alphaproteobacteria*	13 b	10 b	41 a	44 a	16 b	124
OTU005_*Actinobacteria*	40 a	18 b	29 ab	33 ab	0 c	120
OTU006_*Actinobacteria*	23 ab	31 a	22 ab	16 ab	5 b	97
OTU007_*Alphaproteobacteria*	21 ab	37 a	10 bc	19 ab	0 c	87
OTU008_*Alphaproteobacteria*	33	16	13	12	12	86
OTU009_*Actinobacteria*	17	12	23	17	10	79
OTU0010_*Alphaproteobacteria*	13	11	21	8	19	72
OTU0011_*Actinobacteria*	12	10	20	19	11	72
OTU0012_*Actinobacteria*	21	8	18	12	7	66
OTU0013_*Actinobacteria*	16	12	17	12	3	60
OTU0014_*Actinobacteria*	9 b	3 b	4 b	5 b	39 a	60

The most abundant OTUs with sizes ≥60 after normalizing the number of sequences to 4644 per library are presented. OTUs were formed at D=0.03; data with different letters in each row are significantly different, according to SNK-test at P<0.05

[a] OTUs taxonomic affiliations were obtained from the SILVA database

[b] Total number of sequences in an OTU

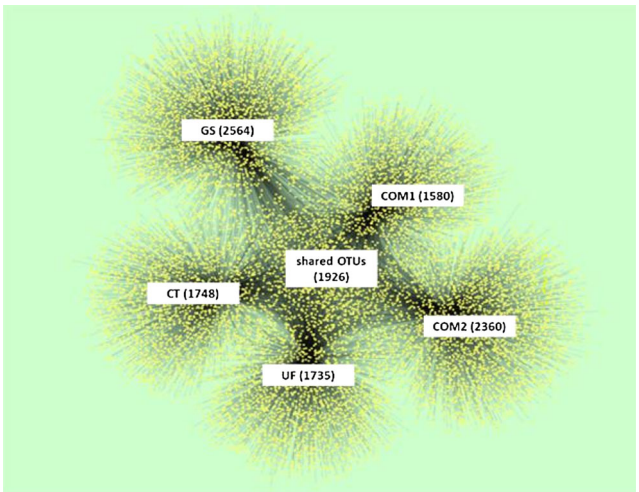

Fig. 3 OTUs network depicting all identified OTUs, number of OTUs per sample and shared OTUs. Each node (*yellow dot*) represents a bacterial OTU. Connections (*black lines*) were drawn between samples and OTUs, with edge weights defined as the number of sequences from each OTU that occurred in each sample. Network was visualized using Cytoscape v3.0.2. *CT* chemical fertilized, *COM1* single dose compost amended, *COM2* double dose compost amended, *UF* unfertilized, *GS* grassland soil

According to a comprehensive review of the dominant soil bacterial taxa in 16S rRNA gene libraries (Janssen 2006), members of the phylum *Proteobacteria* range from 10 to 77 % of abundance. In the bacterial communities studied here, they represented the second most abundant phylum. Although the *Proteobacteria* were in lower abundance in the COM1 and GS soils than COM2 soil, the composition of *Proteobacteria* communities of the COM1 soil closely resembled that of COM2 soil and was very different from that of grassland soils. These results are consistent with the well-known metabolic diversity of this group (Lee et al. 2008).

Similarly to Roesch et al. (2007) and Acosta-Martínez et al. (2008), members of the *Bacteroidetes* were more abundant in agricultural soils than the undisturbed, grassland soils. As all managed soils (UF, CT, COM1, and COM2) differ to the grassland for the presence of sorghum, the observed distribution of *Bacteroidetes* is very likely affected by the presence of plant more than by the adopted management.

The specific sorghum rhizospheric environment may have been also responsible for the differences in *Acidobacteria* abundance. Bacteria belonging to the phylum *Acidobacteria* have been observed in a wide variety of environments (Lee

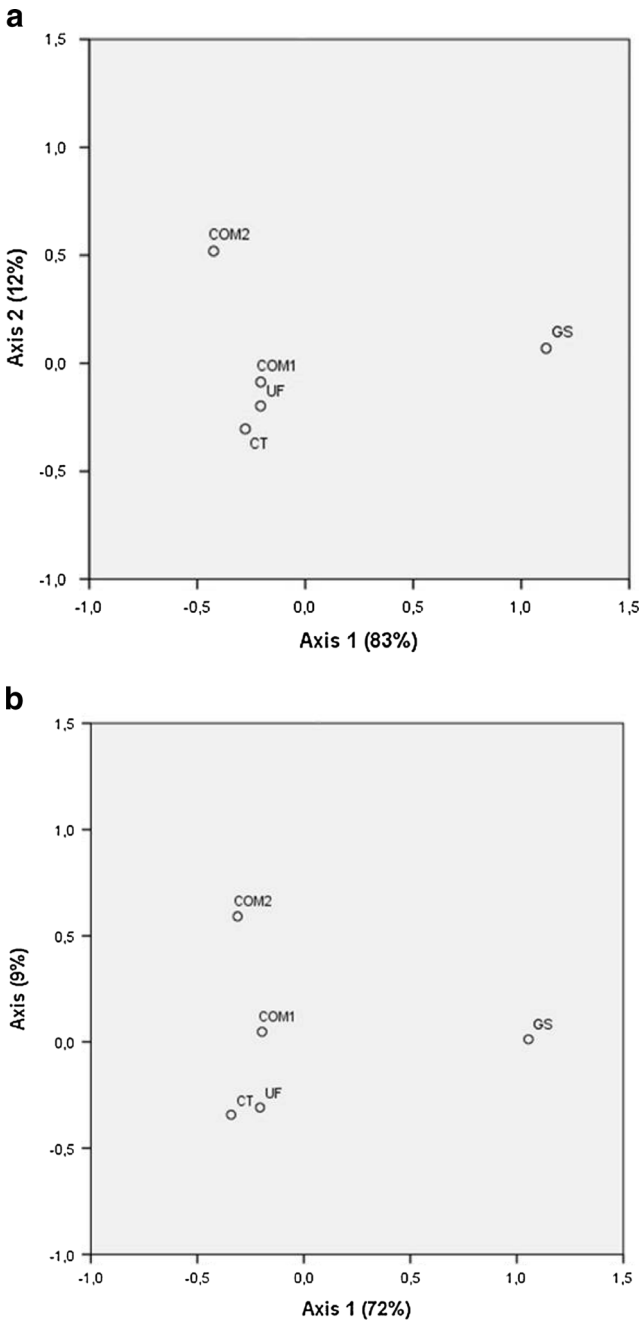

Fig. 4 PCoA plots based on **a** weighted and **b** unweighted Unifrac distance matrices showing the quantitative and qualitative clustering of samples. *CT* chemical fertilization, *UF* not fertilized, *COM2* double dose compost amendment, *COM1* single dose compost amendment, *GS* grassland soil

et al. 2008). However, despite the ubiquity and abundance of these bacteria, our knowledge of this phylum is limited because relatively few species have been characterized. Nevertheless, similar to most of the investigations on the composition of soil bacterial communities (LaPara et al. 2000; Layton et al. 2000; Smit et al. 2001; Martiny et al. 2003), the *Acidobacteria* are less abundant in the sorghum rhizosphere soil than in grassland soil.

Based on the distribution of 16S rRNA gene sequences among the bacterial phyla found in the literature, Smit et al. (2001) suggested that the ratio between the number of *Proteobacteria* and *Acidobacteria* might be determined by the trophic level of soil. Marilley et al. (1999) found that the rhizosphere, which is a relatively copiotrophic niche for bacteria, has a selective effect toward the phylum *Proteobacteria* and is detrimental to the phylum *Acidobacteria*. Strictly correlated to the trophic environment is the soil pH. Most molecular studies have found that pH also influence *Acidobacteria* abundance, with the highest abundances found in environments with the lowest pH (Fierer et al. 2007; Männistö et al. 2007; Lauber et al. 2008). Jones et al. (2009) remarked on this aspect and in a comprehensive survey of soil *Acidobacteria* diversity using pyrosequencing and clone library analyses showed that decreases in soil pH significantly correlated with the relative abundance of *Acidobacteria*.

Previously, it had been shown that organic fertilization stimulates microbial growth in soil (Hartmann et al. 2006; Esperschütz et al. 2007; Bastida et al. 2008; Chaudhry et al. 2012). To better understand which part of the complex soil community responds to organic matter applications, our investigation was deepened to the lower phylogenetic groups. Data relative to the most represented classes of *Proteobacteria* (α, β, δ, γ) showed, as observed for the corresponding phylum, that all classes were lowered to advantage of *Actinobacteria* class, in both compost amendment soils.

When, therefore, the families of the most abundant classes, *Actinobacteria class* and *α-Proteobacteria* were investigated, we found a higher number of *Proteobacteria* families over those belonging to *Actinobacteria*. By a deeper analysis, we were able to determine which bacterial families differentiate compost amended from chemical fertilized plots and other soil managements, as well as to confirm, as reported by Crecchio et al. (2001), that the dose of the compost did not affect relevantly the composition of bacteria. Furthermore, very interestingly, we observed that grassland soil plots (GS) were characterized by a very low level of abundance of some families and a high level of others, resulting thus in a clear separation from all managed cropland. Very likely, these differences could reflect the populations more closely associated with the sorghum roots.

Differences among samples were clearly showed also after the investigation of bacterial diversity at the species level, as indicated by the high number of unique OTUs per sample (species level) compared to the number of shared ones (Fig. 3) and by the statistically significant differences existing for the most abundant species among differently treated plots (Table 4). So, only deepening our investigation to the family and species level, we were then able to evidence some particular bacterial composition differences among the different soil managements. The evaluation of beta diversity by UniFrac gave, lastly, an interesting picture of the bacterial composition

under different fertilization regimes: grassland soil clearly separates from all the others; furthermore, in agreement with Wu et al. (2011), Chaudhry et al. (2012) and Saison et al. (2006), the compost amendment, in particular with a double dose (COM2) changes relevantly bacterial diversity while chemical fertilization did not.

Conclusions

The necessity to adopt sustainable fertilization and crop managements systems has increased with larger demands for increased productivity. As soil bacteria respond more rapidly than other soil components, their characterization is important to understanding the impact of different practices. Many factors could be the cause of the highlighted differences found respect to other studies, such as the different soil conditions, the type of crop used, the type of compost (green waste, sewage sludge, manure, urban organic waste), the analytical approach adopted and the few information still available on most of phyla, and their correlation with specific environments.

Our analysis on the bacterial composition and distribution in the different fertilization regimes studied and the specific approach used have clearly evidenced two aspects. First, the organic fertilization by compost, more than chemical fertilization by urea, affects significantly the composition of bacteria inhabiting sorghum rhizosphere, when compared to unfertilized soil and grassland controls. Of course, these results could at least in part be explained by a kind of "inoculum" effect by the microflora inhabiting compost; nevertheless, it is worth to be considered that a considerable laps of the time occurred between the last amendment and soil sampling. Second, the in-depth analysis of alpha (up to species level) and beta diversity is very important to detect such changes (i.e., effect of double dose of compost) that we observed only up to species level.

Considering these findings and the still limited information available on the effects of sorghum crop on soil microbial community composition, we believe that our study would be useful for other investigations. Important aspects such as the plant impact or the direct or indirect effect of compost on the soil microbial assemblage are, in fact, still to be addressed and may complete our results and provide useful information not only on the best management for the sorghum crop in Mediterranean soils but also for the sustainable management of these soils.

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Conflict of interest The authors declare that they have no conflict of interest.

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