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Soil metagenomics reveals differences under conventional and no-tillage with crop rotation or succession

Renata Carolini Souza[a,b], Mauricio Egídio Cantão[c], Ana Tereza Ribeiro Vasconcelos[d], Marco Antonio Nogueira[a,b], Mariangela Hungria[a,b,*]

[a] Embrapa Soja, Cx. Postal 231, 86001-970 Londrina, Paraná, Brazil
[b] Universidade Estadual de Londrina, Dept. Microbiologia, Cx. Postal 60001, 86051-990 Londrina, Brazil
[c] Embrapa Suínos e Aves, Cx. Postal 21, 89700-000 Concórdia, Santa Catarina, Brazil
[d] Laboratório Nacional de Computação Científica, Rua Getúlio Vargas 333, 25651-071 Petrópolis, Rio de Janeiro, Brazil

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ABSTRACT

Soil conservation practices are critical for agricultural sustainability, and in this study the shotgun sequencing approach was used to investigate the effects on soil biodiversity of different soil- and crop-management practices in a 13-year field trial in southern Brazil. Treatments consisted of conventional tillage (CT) with plowing and disking, or no-tillage (NT) with direct sowing into the residues of previous crops, in a crop succession [soybean (summer)/wheat (winter)] or rotation [soybean/maize (summer)/wheat/lupine/oat (winter)]. About 1 million reads per treatment revealed very high levels of diversity. The majority of the sequences were attributed to the Bacteria (54%), and 0.3% and 0.2% fitted into Archaea and Eucarya domains, respectively; 46% showed no similarity with any known sequences. Major differences were associated with tillage and, to a lesser extent, with crop management. Statistically significant higher abundances with CT encompassed microorganisms associated with residue decomposition, carbon and nitrogen cycling, and xenobiosis. Eucarya were also more abundant with CT, possibly related to higher tolerance of environmental stresses. In contrast, NT showed higher abundances particularly of nitrogen-fixing Rhizobiales and Archaea that inhabit environments rich in organic matter.

1. Introduction

A recent assessment of the state of the planet's land resources shows that overall, 25% and 8% are considered to be highly and moderately degraded, respectively; soil degradation is mostly related to poor agricultural management practices and improper use of fertilizers and pesticides (FAO, 2012). Biotic activity is fundamental to prevent or reverse soil degradation, since microbial communities participate in critical processes, such as the biogeochemical cycles. However, anthropogenic activities can affect both the functionality and the biodiversity of microbial communities, potentially resulting in curtailment of microbial functions and loss of species (Brown et al., 2002; FAO, 2012). Therefore, the global adoption of soil conservation practices in agriculture is critical to help to reverse soil degradation, and to maintain soil fertility and soil biodiversity.

In contrast to conventional agricultural practices of soil management using plowing (0–20 cm) and disking (0–8 cm), the system known as no-tillage is characterized by sowing directly into the residues of previous crops. It has been broadly shown that no-tillage can contribute significantly to soil conservation, mainly by the presence of crop residues on the surface, ameliorating adverse temperature and moisture conditions (Derpsch et al., 1991), increasing soil organic matter content, and improving soil physical properties (Calegari et al., 2008; Castro Filho et al., 2002; Derpsch et al., 1991; FAO, 2012; Franchini et al., 2007; Lal et al., 2007). Most microbial activity occurs within a few centimeters of the soil surface (Babujia et al., 2010); therefore, an increasing number of reports shows that lack of soil disruption and coverage by plant residues in the no-tillage system also favor increased microbial biomass and activity (Babujia et al., 2010; Franchini et al., 2007; Kaschuk et al., 2010; Pereira et al., 2007; Silva et al., 2010, 2013). Estimates indicate that no-tillage systems are practiced on over 117 million hectares worldwide, 48% of which in South America (FAO, 2012). Brazil is a world leader with over 25.5 million hectares under no-tillage,

* Corresponding author at: Embrapa Soja, Caixa Postal 231, 86001-970 Londrina, Paraná, Brazil. Tel.: +55 43 33716206; fax: +55 43 33716100.
E-mail addresses: renata.kerolini@hotmail.com (R.C. Souza), mauricio.cantao@embrapa.br (M.E. Cantão), atrv@lncc.br (A.T.R. Vasconcelos), marco.nogueira@embrapa.br (M.A. Nogueira), mariangela.hungria@embrapa.br, hungria@pq.cnpq.br, hungria@cnpq.br, biotecnologia.solo@hotmail.com (M. Hungria).

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which is expected to increase to 33 million hectares in the next years (FEBRAPDP, 2012).

Avoiding monoculture is another practice critical to diminishment of soil degradation, and rotations/associations should involve at least three different crops (FAO, 2012). Unfortunately, monocultures or successions involving only two crops prevail worldwide – including the southern region of South America – and can deplete organic matter content, result in predisposition to diseases, and increase weed infestation. In contrast, crop rotations, especially with green manures, break pathogen cycles and improve soil physical and chemical properties, including soil organic matter, as has been shown in Brazil (Boddey et al., 2010; Calegari et al., 2008; Derpsch et al., 1991; Franchini et al., 2007). However, the complexity of results obtained with various crop rotations, including the unexpected absence of differences in microbial biomass and activity in some reports in Brazil (Balota et al., 1998, 2004; Franchini et al., 2007; Hungria et al., 2009; Silva et al., 2010, 2013), clearly shows the need for more research to improve understanding of how microbial communities are affected by crops.

In the last decade, a variety of molecular tools has fostered advances in our knowledge of diversity and composition of soil microbial communities, including denaturing gradient gel electrophoresis (DGGE) (Helgason et al., 2010; Kozdrój and van Elsas, 2001; Muyzer et al., 1993; Peixoto et al., 2006) and fatty acid signatures (Hedlund, 2002; Helgason et al., 2010; Kozdrój and van Elsas, 2001). Significant advances are now possible with DNA-sequencing analyses, with emphasis on metagenomics (Delmont et al., 2012; Roesch et al., 2007). However, studies of metagenomes using environmental shotgun sequencing (ESS) – instead of the sequencing analysis of cloned libraries or specific genes such as that for 16S rRNA – are still rare.

Our understanding of microbial communities in intensively cropped areas of South America is still poor, and metagenomics offer the promise of revealing diversity under varied soil and management systems. In this study, a metagenomic approach was adopted for examination of an Oxisol under no-tillage or conventional tillage conditions with crop succession (soybean/wheat) or rotation (five crops in a 5-year period) practices in a long-term field trial in Brazil.

2. Materials and methods

2.1. Description of the field trial and treatments

The study was performed in a long-term experiment, established in the summer of 1997/1998 at the experimental station of Embrapa Soja, in Londrina, State of Paraná, Brazil, latitude 23°11′ S, longitude 51°11′ W, and elevation of 620 m. The soil is classified as Latossolo Vermelho Eutroférrico (Brazilian system), Rhodic Eutrudox (US taxonomy) and main chemical and physical properties are given in Table 1. Climate conditions have been given elsewhere (Silva et al., 2010).

The treatments consisted of no-tillage (NT) and conventional tillage (CT), each under a crop succession (CS) [soybean (*Glycine max* L. Merr.) in the summer and wheat (*Triticum aestivum* L.) in the winter] or a crop rotation (CR) with soybean and maize (*Zea mays* L.) in the summer and wheat, lupine (*Lupinus angustifolius* L.) and oat (*Avena strigosa* Schreb.) in the winter. The four treatments are designated as NTS, NTR, CTS and CTR. Crops grown in succession and rotation for the last 7 years are shown in Supplementary Table S1. Each plot measured 8 m width × 15 m length. The trial had a completely randomized block design, with four replicates.

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2.2. Soil sampling and processing

Sampling was performed in early November of 2010, immediately before sowing of soybean, and 3 weeks after harvesting the winter crop, wheat. Crop residues of the wheat were incorporated to the soil in the CT system and left on the soil surface in the NT system. An area of 0.4 m^2 was delineated on each plot with a metal square and the surface 0–10-cm layer was determined. Crop residues were removed and a soil sample of approximately 300 g was taken from the middle of each square using an auger. The samples were placed in labeled bags and the procedure was repeated eight times in each of the four replications in the field, at points spatially distributed as representative of the area of each replicate. The discrete soil samples were combined so that each replication consisted of a composite sample of approximately 2.5 kg per plot, with four plots per treatment. In the laboratory, plant residues were removed, the samples were homogenized and passed through a 2-mm sieve before analysis. Subsamples were taken for chemical and physical analyses, and the remaining was kept at −20 °C.

2.3. Homogeneity among replicates

To confirm homogeneity for microbial DNA, each field replicate was first submitted to 16S rRNA analysis by PCR-DGGE, as described before (Silva et al., 2013). The DGGE gels were analyzed using Bionumerics software (Applied Mathematics, Kortrijk, Belgium, v.4.6), with algorithm UPGMA and tolerance of 5% to create a distance matrix, as defined before (Silva et al., 2013). The level of similarity between replicates in each treatment ranged from 98.8 to 100% (data not shown). Therefore, the four replicates of each treatment were considered homogeneous and were pooled to extract the DNA for the metagenomic analysis.

2.4. DNA extraction and pyrosequencing analysis

Metagenomic DNA was extracted by using 10 g of each soil replicate and the PowerMax™ Soil DNA Isolation Kit (MoBio Laboratories), following the manufacturer's procedures. DNA was quantified and purity was verified in a NanoDrop spectrophotometer at 260 and 280 nm. DNA purity and quantity were also verified by electrophoresis in 1% agarose gels and samples adjusted to 50 ng/μL.

Metagenomic DNA was submitted to sequencing analysis in the 454 platform (GS-FLX *Titanium* Roche Applied Science), at the Labinfo of LNCC (Petrópolis, Rio de Janeiro, Brazil http://www.labinfo.br). For the pyrosequencing, DNA was randomly fragmented by nebulization with compressed nitrogen gas. Fragments of 300–800 bp were selected (Bioanalyzer DNAChip) (Jones, 2010). Fragments were then linked to adaptors A and B, according to the manufacturer's procedure and linked to the microsphere for the emulsion PCR (Margulies et al., 2005). Samples were submitted to sequence analysis with the Titanium kit, and the support *PicoTiterPlate* (Roche Applied Science) (Imelfort and Edwards, 2009).

To remove low-quality sequences and artificial duplicated reads (Niu et al., 2010) filters were applied to the sequencing analysis. In order to remove artificial reads and low-quality sequences, Replicates software (Gomez-Alvarez et al., 2009) and Lucy (Chou and Holmes, 2001) were applied to the sequences obtained. The data set was deposited in the NCBI-SRA (Sequence Read Archive) with the submission Accession Number SRA050780.

2.5. Data analyses

Sequences were analyzed with the MEGAN4 software (Huson et al., 2011), based on the comparison of BLASTX with all the

Table 1
Soil chemical and granulometric[a] properties in the 0–10 cm layer.

Soil management[b]	Crop management[c]	pH	Al	H + Al	K	Ca	Mg	SB[d]	T_{CEC}[d]	P	C	N	BS[d]
		$CaCl_2$	($cmol_c\ dm^{-3}$)							($mg\ dm^{-3}$)	($g\ dm^{-3}$)	($g\ dm^{-3}$)	%
NT	CS	5.39[e]	0	4.67	0.53	4.03	1.24	5.79	10.47	66.0	24.0	2.6	55.00
NT	CR	5.68	0	3.95	0.58	4.50	1.20	6.28	10.22	55.5	25.8	2.8	61.24
CT	CS	5.23	0	4.77	0.46	3.73	1.01	5.20	9.97	14.8	19.2	1,6	52.11
CT	CR	5.32	0	3.91	0.47	4.06	1.15	5.68	9.60	20.6	19.4	1.8	59.22

[a] Granulometric composition: 710 $g\ kg^{-1}$ of clay, 82 $g\ kg^{-1}$ of silt and 208 $g\ kg^{-1}$ of sand.
[b] Conventional tillage (CT), no-tillage (NT).
[c] Crop succession (CS) with soybean (summer) and wheat (winter) and crop rotation (CR) with soybean and maize (summer), and wheat, lupine, and oat in the winter, as described in Table 1S.
[d] SB, sum of bases (Ca + Mg + K); T_{CEC}, total cation exchange capacity (H + Al + Ca + Mg + K); BS, base saturation (SB/T_{CEC}) × 100.
[e] Data represent the means of four field replicates.

sequences in the NCBI-NR databank. In MEGAN, the comparison of the hits and attribution of a taxon for each sequence is based on the lowest common ancestor algorithm (LCA). The parameters used in the software were the min-support: 5; min-score: 60; top-percent: 10. Data were also normalized by using the MEGAN software.

Data were also submitted to statistical analysis with the STAMP software (Parks and Beiko, 2010), to identify differences in taxonomical distribution in the comparison of all combinations of pairs of treatments. Statistical significance was estimated with the Fisher's test for $p \leq 0.05$, using Storey's FDR correction method for comparison of pairs of treatments. The confidence interval was estimated with the method of Newcombe–Wilson.

3. Results and discussion

3.1. Sequencing analysis, data normalization, and rarefaction curves

About 1 million sequences were generated for each of the four treatments: conventional tillage with crop rotation (CTR), 1,050,712 sequences, average size of 304 bp; CT with crop succession (CTS), 1,080,923 sequences of 307 bp; no-tillage with crop rotation (NTR), 913,435 sequences of 306 bp; no-tillage with crop succession (NTS), 1,034,153 sequences of 311 bp. In the assemblage with the Newbler software, very few contigs were generated: CTR, 51, the biggest with 1323 bp; CTS, 83, with 1287 bp; NTR, 31, with 1055 bp; NTS, 58, with 1505 bp.

As the number of reads was similar among the treatments, data were normalized for a set of 100,000 sequences, to facilitate comparisons. Normalization means that the 100,000 sequences were chosen by definition of the software to represent a common number between treatments, but it refers to the 1,000,000 sequences from each treatment that were analyzed. Rarefaction curves generated by the MEGAN for the species level are shown in Supplementary Figure S1. Even with 1 million sequences, the curves were not saturated, indicating a high level of diversity. As a comparison, Havelsrud et al. (2011) obtained 459,262 reads form sea sediments in California, USA, and in the comparison with four soils from USA, Canada and Brazil, Roesch et al. (2007) analyzed a maximum of 53,533 reads per site.

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3.2. Total soil microbial diversity

It is important to highlight that the results shown in our study were obtained with a shotgun approach, reading all fragments and without a previous amplification of ribosomal genes. Therefore, results should represent a much broader diversity than studies based on ribosomal genes. A comparative tree of the four genomes was generated with the MEGAN, and Fig. 1 shows the results obtained at the Phylum level. The majority of the sequences were attributed to the Bacteria domain (53.5%), while 0.3% and 0.2% were attributed to the Archaea and Eucarya domains, respectively, whereas viruses represented only 0.0001%. On the other hand, 46.1% of the sequences did not show any similarity (BLASTX) with any sequence of the NCBI-NR (Fig. 1). Similar proportions of microorganisms in the three domains were reported in another metagenome study of a natural grassland soil at Rothamsted Experimental Station, Harpenden, UK: over 12.5 million reads were obtained, and, based on functional classification of MG-RAST (Meyer et al., 2008) – an even more restricted database – only 34.5% of the reads were annotated, of which 88.6%, 0.91% and 1.41% had closest homologies with Bacteria, Eucarya and Archaea, respectively (Delmont et al., 2012). One possible explanation for the low proportion of Eucarya and Archaea in our study, and in others, may be the unbalanced sequence database, i.e. the much larger number of genomes and sequences of Bacteria. It is possible that most of the sequences classified as "no hits" belong to the Eukcarya and Archae domains, but this remains to be confirmed.

3.3. Bacteria, Archaea and Eucarya domains, and virus

Within the Bacteria domain, the Proteobacteria represented the dominant phylum in all four treatments - 41.5% (Fig. 1). The prevailing classes were Alphaproteobacteria (51.1%), Betaproteobacteria (20.8%), Deltaproteobacteria (19.6%) and Gammaproteobacteria (8.55%) (Fig. 2A). The order Rhizobiales dominated the Alphaproteobacteria (Fig. 2B), with numerous symbiotic nitrogen-fixing bacteria, including the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Mesorhizobium*. Within this order, another agriculturally important genus was *Nitrobacter*, critical for nitrogen cycling. Within the genus *Rhodopseudomonas*, one relevant species was *Rhodopseudomonas palustris*, a phototrophic bacterium that has been used as a model for anaerobic degradation of aromatic compounds (Perrotta and Harwood, 1994). Although mostly aerobic, the soils in our study are rich in clay (Table 1), and pores – with an emphasis on micropores – can become anaerobic for varying periods of time, mainly after rain; furthermore, anaerobiosis can result also from soil compaction caused by excessive traffic, at least in the topsoil.

Sphingomonadales represented the second most abundant order of Alphaproteobacteria (Fig. 2B), with predominance of the genus *Sphingomonas*. Among other functions, these bacteria may act in the mineralization of herbicides (Sørensen et al., 2001) and in our experiment, in both CT and NT systems, herbicides have been heavily applied. In the order Caulobacterales (Fig. 2B), the most abundant genus was *Caulobacter*, including bacteria involved in carbon cycling and capable of surviving in oligotrophic environments (Marks et al., 2010). Other hits fit into the order Rhodospirillales (Fig. 2B), including *Azospirillum*, which may give important contributions to plant growth due both to nitrogen fixation and

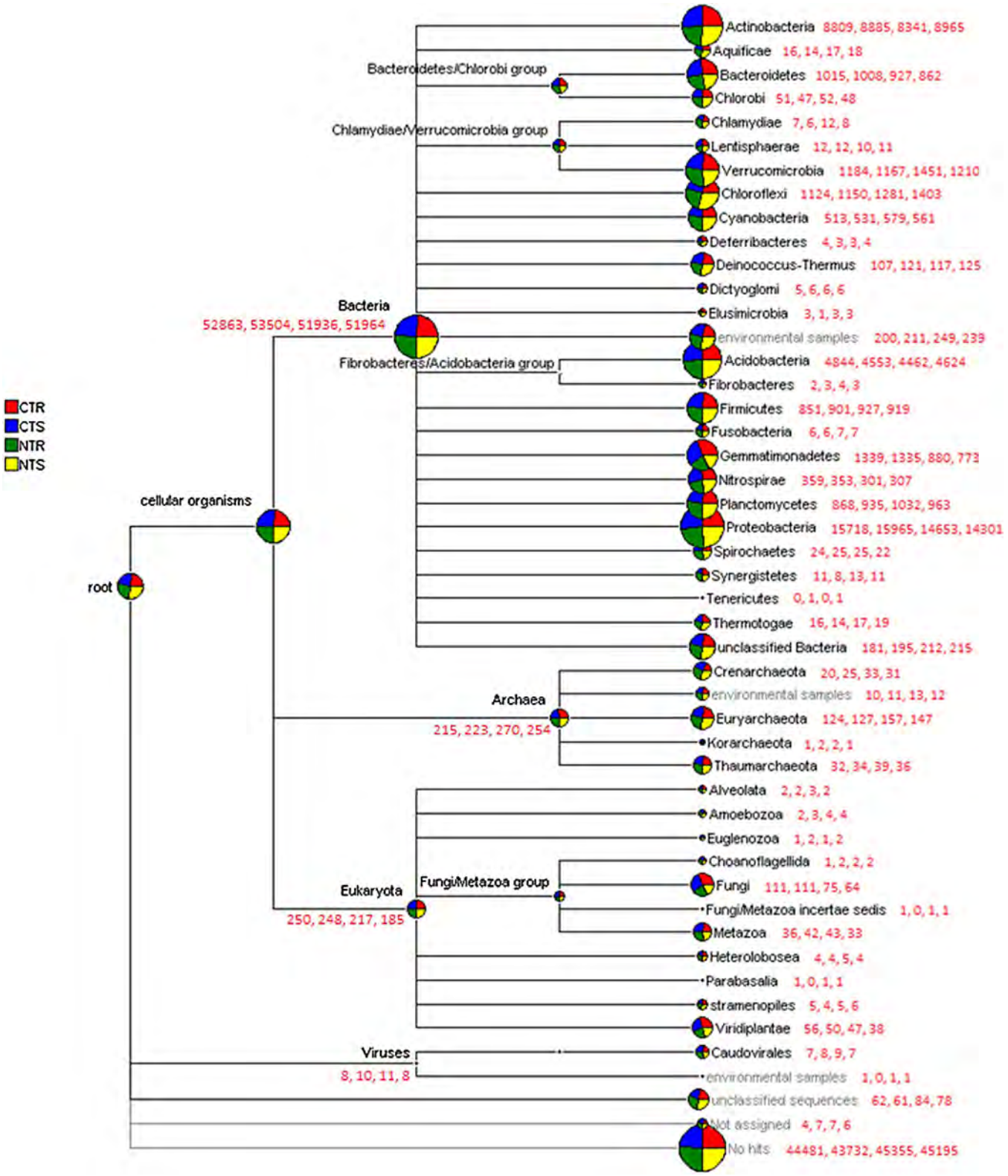

Fig. 1. Comparative tree generated by the MEGAN software of four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

plant-growth promotion via the biosynthesis of hormones (Hungria et al., 2010; Steenhoudt and Vanderleyden, 2000).

The Betaproteobacteria class was the second most abundant of the Proteobacteria (Fig. 2A), with the great majority of hits in the order Bukholderiales, followed by Neisseriales, Nitrosomonadales and Rhodocyclales (Fig. 2C). *Burkholderia* has a variety of functions in soils, including biological control of pathogens, bioremediation of xenobiotics (Coenye and Vandamme, 2003), plant-growth promotion (Estrada-De Los Santos et al., 2001), and nitrogen fixation (Gyaneshwar et al., 2011). The order Nitrosomonadales included

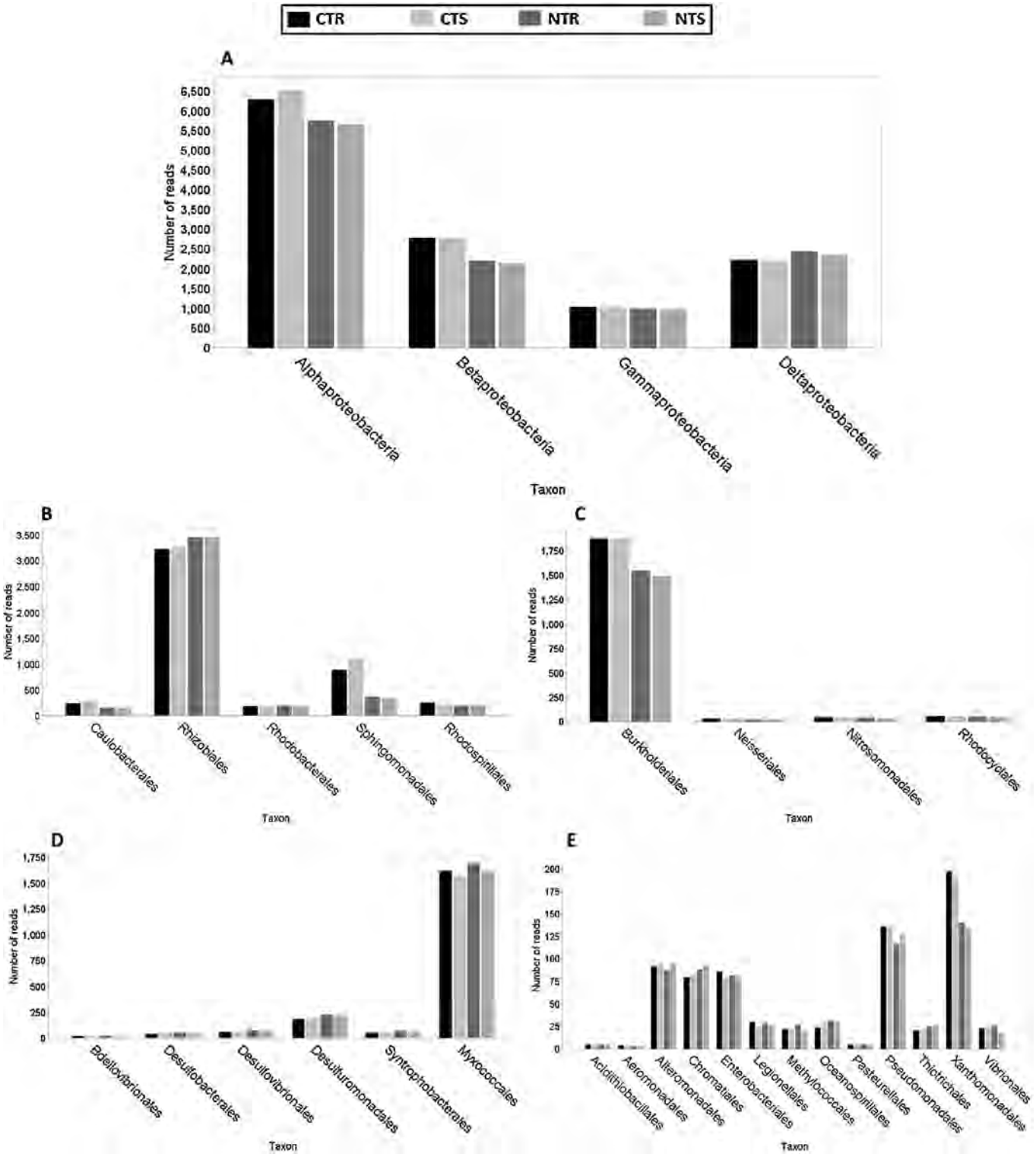

Fig. 2. Abundance estimates generated by the MEGAN software of (A) classes of the Phylum Bacteria, and orders of (B) the class Alphaproteobacteria, (C) Betaproteobacteria, (D) Deltaproteobacteria, (E) Gammaproteobacteria in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

several chemolithotrophic genera, including *Nitrosomonas* and *Nitrosospira*, related to nitrification (Schmidt and Bock, 1997; Shaw et al., 2006).

The number of hits with Deltaproteobacteria was similar to that of Betaproteobacteria (Fig. 2D), and bacteria belonging to these orders participate in iron and sulfate reduction (Foti et al., 2007; Hori et al., 2010), and may have important roles in the availability of these nutrients for both plants and soil microorganisms. Finally, in the Gammaproteobacteria class (Fig. 2A) the four most abundant orders were Xanthomonadales, Pseudomonadales, Alteromonadales and Chromatiales (Fig. 2E), with the genera *Xanthomonas*, *Pseudomonas*, *Marinobacter* and *Nitrosococcus*, respectively.

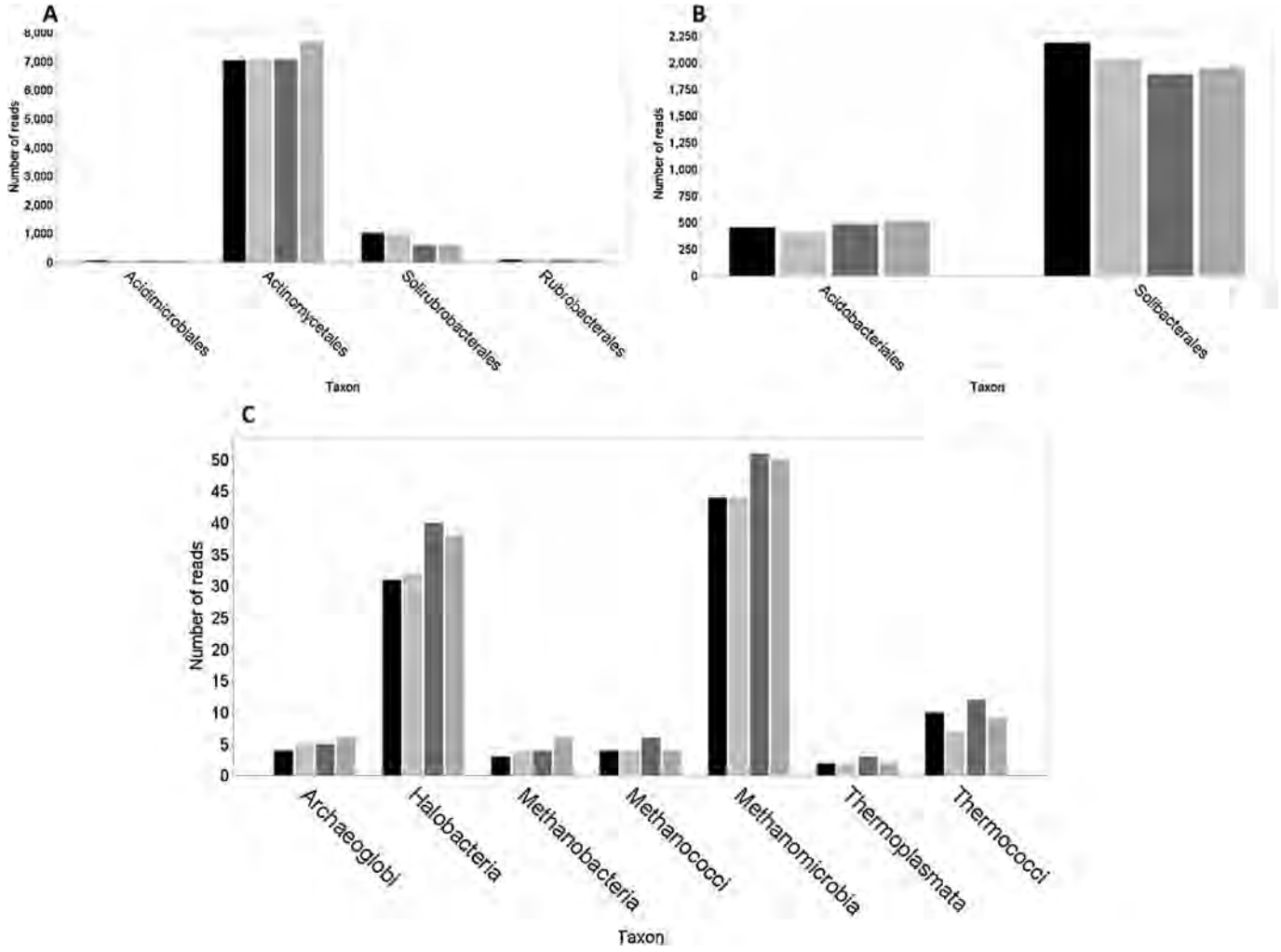

Fig. 3. Abundance estimates generated by the MEGAN software of (A) phylum Actinobacteria, (B) order Acidobacteria, (C) classes of the phylum Euryarchaeota in four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

Xanthomonas spp. are usually plant pathogens capable of surviving under limiting nutrient conditions, being strong competitors in soils (Habte and Alexander, 1975). *Pseudomonas* spp. are characterized by high versatility in nutrient requirements; the species participate in several important processes, including nitrogen cycling, degradation of aromatic compounds (Palleroni, 2009), and plant-growth promotion (Saharan and Nehra, 2011).

Actinobacteria was the second most abundant phylum of bacteria (24.0%) (Fig. 1), and the main orders were Actinomycetales, Solirubrobacterales and Rubrobacterales (Fig. 3A). The Actinomycetales are biotechnologically important due to the production of antibiotics by *Streptomyces* (Schlatter et al., 2009), also abundant in our study. Another genus with several hits was *Frankia*, a nitrogen-fixing symbiont with actinorhizal genera *Alnus* and *Casuarina* (Chaia et al., 2010). The phylum Acidobacteria was the third most abundant (12.7%) (Fig. 1), with the orders Solibacterales and Acidobacteriales (Fig. 3B). Other phyla with several hits were the Verrucomicrobia (3.45%), Choloflexi (3.40%), Gemmatimonadetes (2.98%), Bacteroidetes (2.60%), Planctomycetes (2.60%), Firmicutes (2.48%), and Cyanobacteria (1.50%) (Fig. 1). Verrucomicrobia are important members of the rhizosphere, and have been isolated from a variety of plant species, e.g. from *Pinus contorta* (Chow et al., 2002). The role of these microorganisms in the rhizosphere is poorly understood, but there are reports of methane oxidation (Dunfield et al., 2007; Pol et al., 2007). Choloflexi include bacteria with an important role in the decomposition of organic matter (Yamada et al., 2005). In the Gemmatimonadetes the only species detected was *Gemmatimonas aurantiaca* (data not shown); the first member of this phylum was recently described and recognized as a polyphosphate-accumulating microorganism (Zhang et al., 2003). Brazilian Oxisols are usually limited by phosphorus and the P contents in our experiment were not high (Table 1); therefore, *G. aurantiaca* might be important in tropical soils. The phylum Bacteroidetes included plant-growth promoting (Soltani et al., 2010) and cellulose-decomposing (Verkhovtseva et al., 2007) bacteria, among other functions. Cyanobacteria are photosynthetic; some are capable of fixing nitrogen and others improve soil-aggregation stability (Issa et al., 2007), a key aspect of soil conservation. In addition, they may contribute to pathogen suppression (Domracheva et al., 2010), and bioremediation of pesticides and toxic metabolites (Cáceres et al., 2008). Some Firmicutes are involved in the biodegradation of hydrocarbons (Das and Mukherjee, 2007), as well as plant-growth promotion (Lima et al., 2011), and in our study included *Bacillus* and *Clostridium*. The phylum Nitrospirae hosts nitrite oxidizers (Attard et al., 2010; Lücker et al., 2010), and Planctomycetes participate in the oxidation of ammonia (Fuerst and Sagulenko, 2011).

Independently on the method of analysis, soil metagenomes have shown predominance of the phyla Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi,

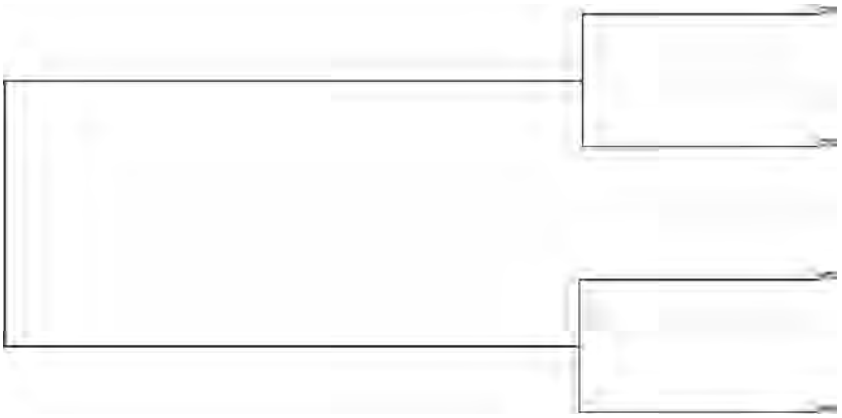

Fig. 4. Cluster analysis based on Euclidean distance and generated with the MEGAN software with the UPGMA algorithm for four metagenomes of an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession (S) or rotation (R).

Planctomycetes, Gemmatimonadetes and Firmicutes, with an emphasis on the Proteobacteria (Delmont et al., 2012; Janssen, 2006; Roesch et al., 2007; Yin et al., 2010). Our data show similar results in a Brazilian Oxisol, but some differences were detected in the less-abundant phyla. For example, in contrast to the results of our study, pyrosequencing of the 16S rRNA indicated greater abundance of Bacteroidetes in four soils, including one from Brazil, whereas Verrucomicrobia were not detected (Roesch et al., 2007).

Few sequences were classified in the Archaea domain, with the following main orders: Euryarchaeota, Thaumarchaeota, Crenarchaeota and Korarchaeota (Fig. 1). In the Euryarchaeota, the classes Methanomicrobia, Halobacteria and Thermococci predominated (Fig. 3C). Methanomicrobia includes methanogenic archaeans, in general present in environments rich in organic matter (Pazinato et al., 2010); the other phyla were less abundant. Within the Thaumarchaeota are microorganisms that participate in the cycling of nitrogen by nitrification, and of carbon by CO_2 fixation (Pester et al., 2011). The non-thermophile Crenarchaeota are also found in environments rich in organic matter and some colonize roots of plants, but their function is not well understood (Simon et al., 2000).

Not many sequences were attributed to the Eucarya domain, and the kingdoms Fungi, Metazoa and Viridiplantae predominated (Fig. 1). In the Fungi, the most abundant phylum was of the Ascomycota, with a variety of saprophytes and endophytes associated with plant roots. There were sequences of species belonging to the order *Sordariales*, including *Chaetomium globosum* (class Sordariomycetes), an antibiotic producer that suppresses pathogens (Di Petro et al., 1992), *Podospora anserina*, which degrades complex carbon compounds, such as lignin and cellulose (Espagne et al., 2008), and *Sordaria macrospora*, a model fungus of biotechnological interest (Nowrousian et al., 2010). Another genus was *Aspergillus* (class Eurotiomycetes), which includes decomposers of soil organic matter (Omemu et al., 2005) and phosphate solubilizers (Asea et al., 1988). In Oxisols of the Brazilian Cerrados, Castro et al. (2008) also described an abundance of Sordariomycetes.

Viral sequences were few, and fit into the order Caudovirales (Fig. 1). These are phages with double-stranded DNA that infect bacteria and some archaeans, affecting the abundance and composition of bacterial communities (Swanson et al., 2009).

The number of sequences attributed to each taxon was compared between the treatments based on a Euclidean matrix of distance, estimated with the UPGMA algorithm and represented as a phylogenetic tree. Two main clusters were observed, for the NT and the CT systems, clearly indicating differences in microbial-community structure resulting from the absence or presence of plowing and disking practices (Fig. 4). Within each soil-management system, two subclusters were formed, related to crop rotation and crop succession (Fig. 4). Therefore, an effect of crop management on diversity was also confirmed, but it was weaker than the soil management effect.

Using the STAMP software, the majority of differences observed in the analyses with MEGAN4 were confirmed. The most abundant phylum – Proteobacteria – was statistically larger under CT, related to the Alpha and Betaproteobacteria classes, whereas the Deltaproteobacteria were more abundant in the NT system (Fig. 2A, statistics not shown). Among the main orders of Proteobacteria that were statistically more abundant with CT, both under crop rotation (Fig. 5) and succession (Fig. 6), were the Sphingomonadales, Caulobacterales (Alphaproteobacteria), Burkholderales, Neisseriales, Nitrosomonadales, Rhodocyclales (Betaproteobacteria), and Xanthomonadales (Gammaproteobacteria). Some orders were more abundant with CT, but only under rotation or succession, e.g. Pseudomonadales (Gammaproteobacteria) in the CTS in comparison to the NTS. The order Solirubrobacterales of the phylum Actinobacteria was also more abundant under CT (Figs. 5 and 6).

3.4. Main differences associated with soil and crop management

Ploughing and disking in the CT system break up and incorporate crop residues into the surface layers of the soil. Decomposition and mineralization are then accelarated, especially under tropical conditions, resulting in prompt increases in microbial respiration and mineralization, and following decreases in microbial carbon and nitrogen contents (Franchini et al., 2007; Hungria et al., 2009). All orders of Proteobacteria that were statistically more abundant in the CT system are functionally related to the decomposition of organic matter, and carbon and nitrogen cycling – with an emphasis on mineralization and denitrification – and degradation of xenobiotics. It is, therefore, plausible that the microbial community has been selected to decompose and mineralize the residues that are incorporated into the soil at the end of each crop season. It should be remembered that several studies have shown a rapid increase of bacterial biomass after incorporation of fresh plant residues (e.g. Fierer et al., 2007; Franchini et al., 2007; Lundquist et al., 1999); specifically Fierer et al. (2007) have shown that the addition of easily degradable organic C was significantly correlated with the abundance of Alpha and Betaproteobacteria, what would support the results from our study. However, we must also remember that with time, carbon and nitrogen stocks decrease in the CT (Babujia et al., 2010; Derpsch et al., 1991; Franchini et al., 2007; Hungria et al., 2009; Lal et al., 2007; Wright et al., 2008), and microorganisms that can utilize more effectively a variety of carbon sources are selected. Interestingly, these results are in agreement with a previous study from our group performed in vitro, in which

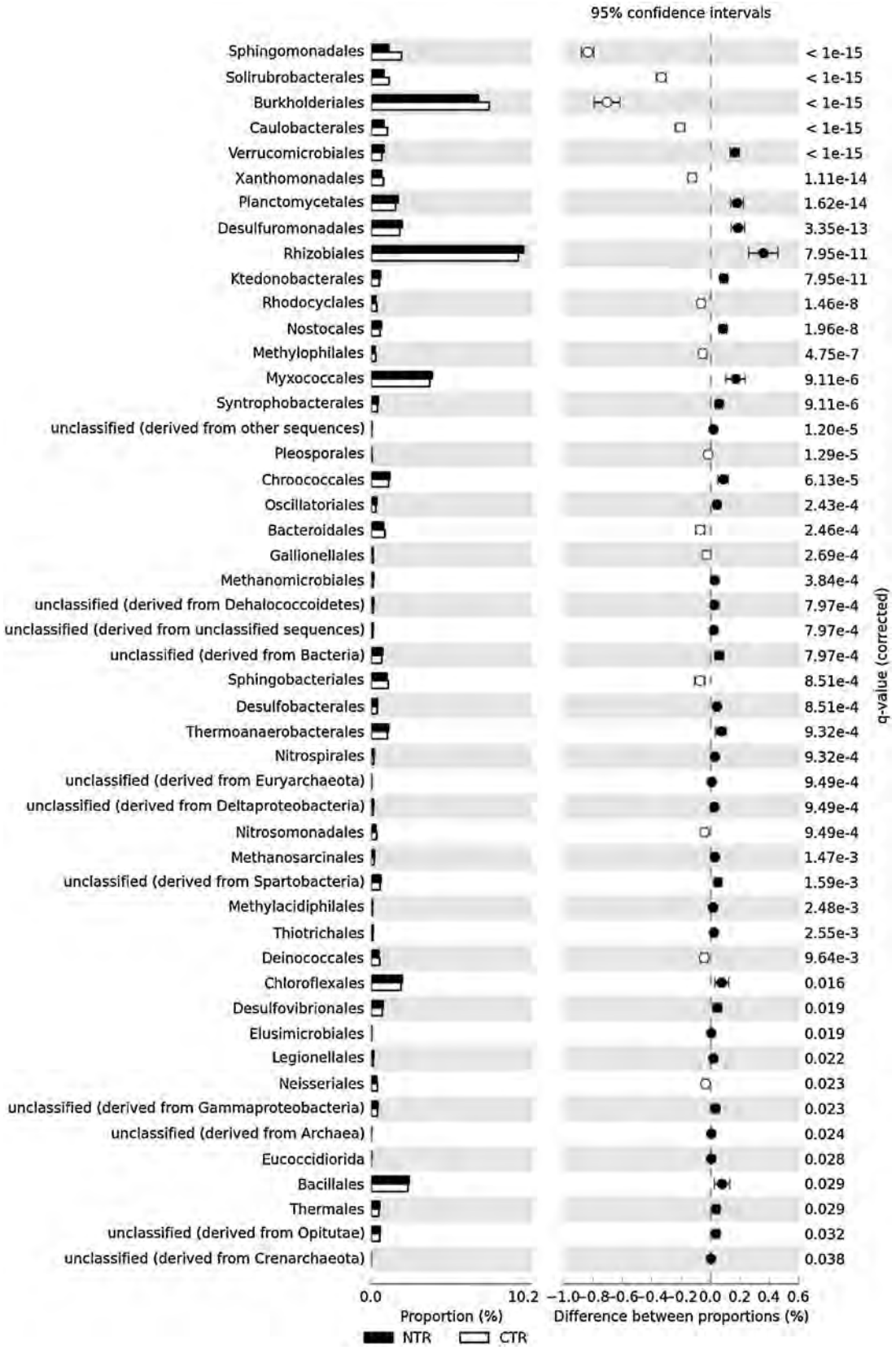

Fig. 5. Statistically significant differences between orders of microorganisms in an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop rotation. The graphic, obtained with the STAMP software, shows the differences between the proportions of sequences in each treatment with a confidence interval of 95%.

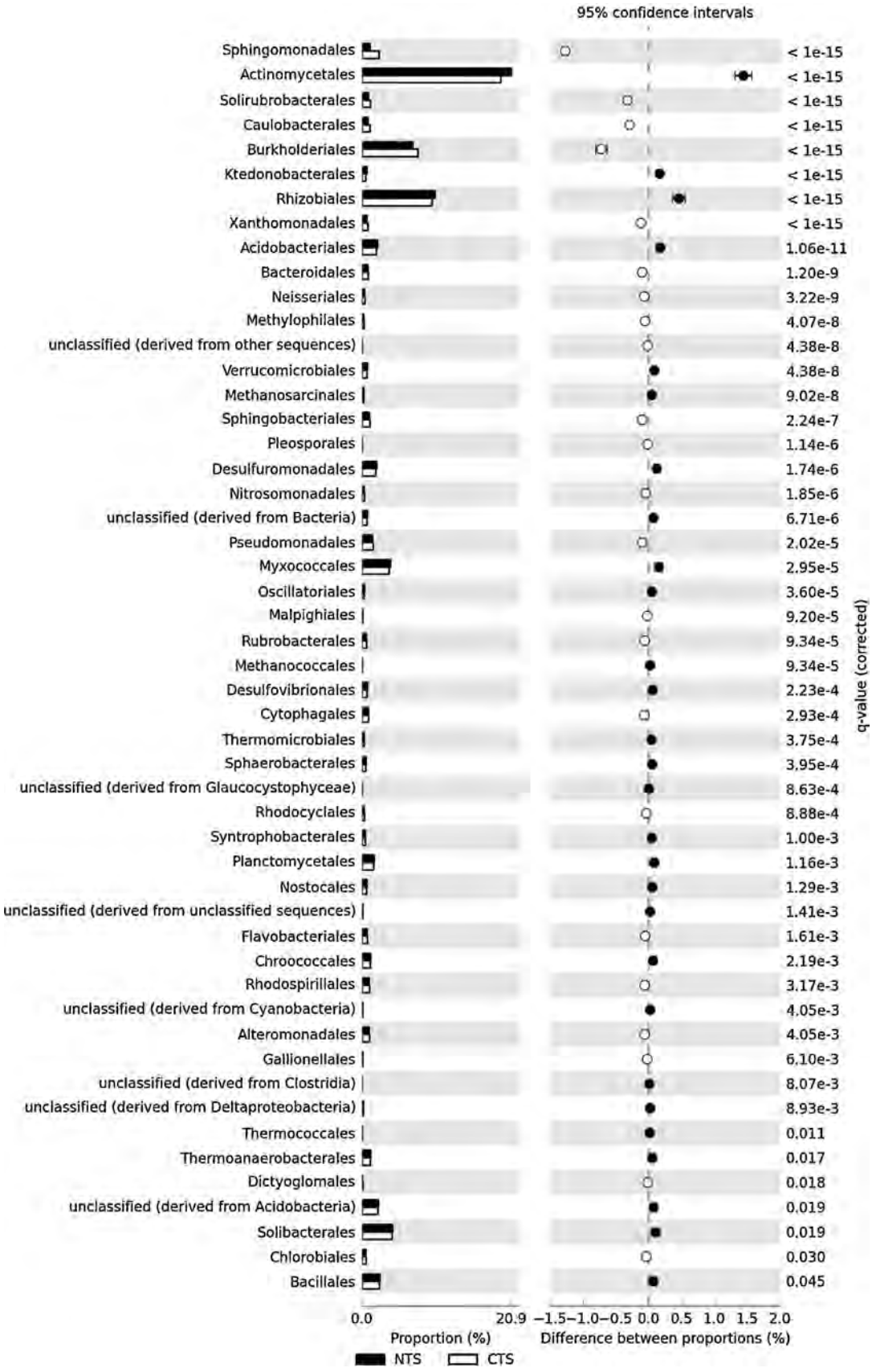

Fig. 6. Statistically significant differences between orders of microorganisms in an Oxisol under 13 years of no-tillage (NT) or conventional tillage (CT) with crop succession. The graphic, obtained with the STAMP software, shows the differences between the proportions of sequences in each treatment with a confidence interval of 95%.

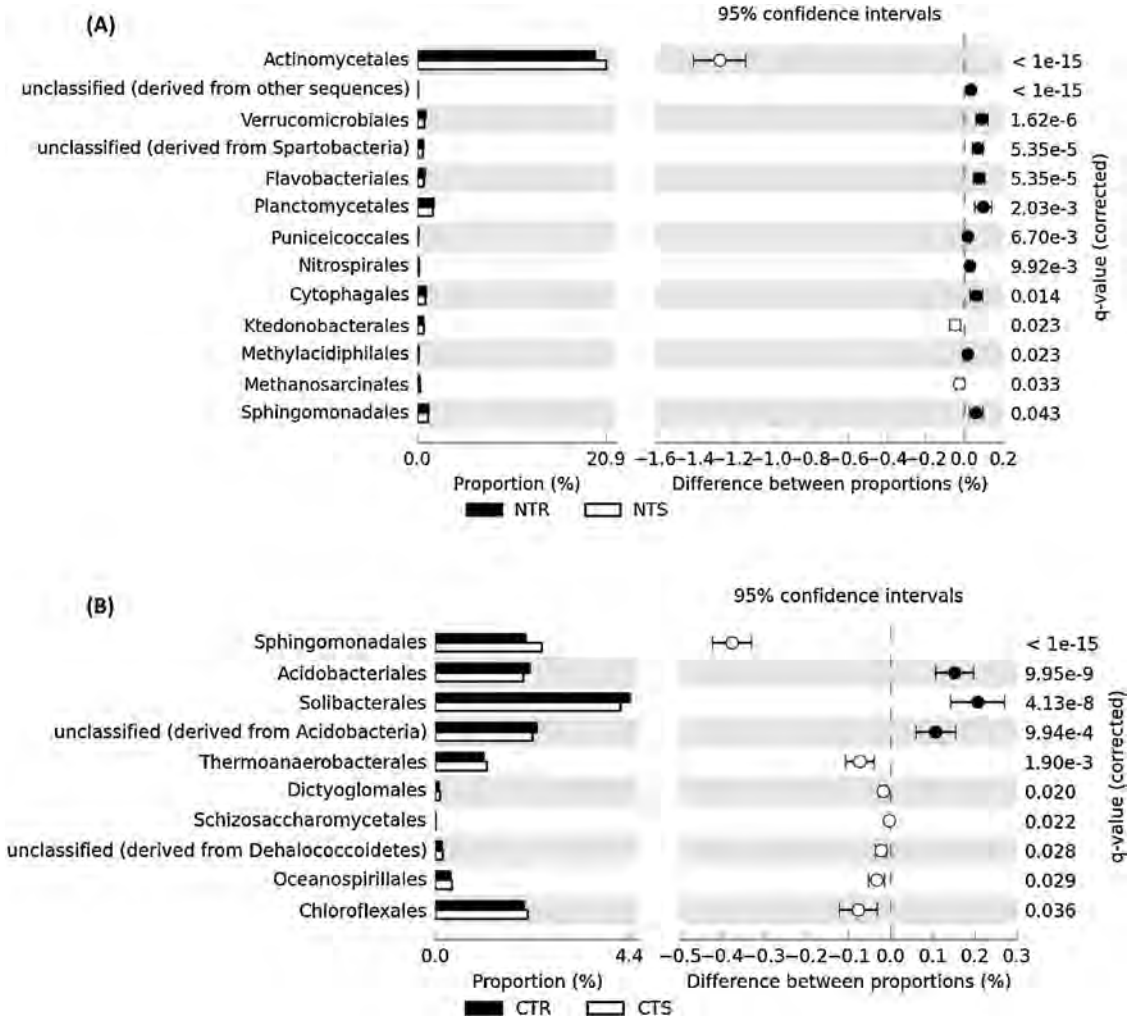

Fig. 7. Statistically significant differences between orders of microorganisms in an Oxisol under 13 years of (A) no-tillage with crop rotation (NTR) or succession (NTS), and (B) conventional tillage with crop rotation (CTR) or succession (CTS). The graphic, obtained with the STAMP software, shows the differences between the proportions of sequences in each treatment with a confidence interval of 95%.

rhizobial strains isolated from the CT system had higher capacity to utilize a broader range of carbon sources than those from the NT system (Hungria et al., 2001). It is also noteworthy that, despite the statistically lower microbial biomass with CT in comparison with NT reported in several studies performed by our group in the same area (e.g. Babujia et al., 2010; Franchini et al., 2007; Hungria et al., 2009; Pereira et al., 2007; Silva et al., 2010), the prevalence of plant pathogens is higher in the former, what could be attributed to bacteria of the orders Xanthomonadales and Pseudomonadales, as observed in this metagenome study. Finally, in our previous study we also showed that rhizobia isolated from the CT system were more tolerant of environmental stresses such as high temperature and salinity (Hungria et al., 2001), indicating that they were adapted to the stressful conditions of the CT system (Derpsch et al., 1991; Hungria and Vargas, 2000). Accordingly, the most abundant orders identified in this study are also tolerant of stresses.

One surprising result was that microorganisms of the Eucarya domain were more abundant in the CT system, mainly the kingdom Fungi. It is general believed that the fungi are more abundant with NT because conventional agricultural practices can disrupt hyphae (e.g. Castro et al., 2008). As we mentioned before, it is premature to draw conclusions about the fungal community, as the number of metagenomic reads of fungi was low. Furthermore, the number of no hits was higher under NT, thus the unknown sequences may hide a large population of fungi. For now, we propose that the greater abundance of fungi observed with CT is related to higher tolerance to environmental stresses, typical of CT systems.

In the NT system, the statistically higher abundance of the Rhizobiales – composed of several nitrogen-fixing rhizobial species – should be emphasized (Figs. 5 and 6). Biological nitrogen fixation is a key process in Brazilian areas planted to soybean and can fullfill most of that legume's N needs (Hungria et al., 2006). Previous reports from our group with microsymbionts both from common bean (*Phaseolus vulgaris* L.) and from soybean have indicated that the number of cells, rhizobial genetic diversity, and the rates of nitrogen fixation were higher under NT than with CT (Ferreira et al., 2000; Kaschuk et al., 2006; Pereira et al., 2007), and higher abundance has now been confirmed using the metagenomics approach. Increased contributions from biological nitrogen fixation under NT have been attributed to better environmental conditions and improvement of soil aggregation (Hungria and Vargas, 2000).

All orders of the Deltaproteobacteria class – Myxococcales, Desulfuromonadales, Desulfovibrionales, Desulfobacterales, and Syntrophobacterales – were also more abundant in the NT system (Figs. 5 and 6). A higher number of hits was attributed to the Myxococcales, and it is possible that the bacteria were favored by the higher organic matter content with NT (Lueders et al., 2006).

Our knowledge about the Archaea domain needs improvement, particularly since the majority of the available information has come from studies of extremophiles. Intriguingly, Archaea were consistently more abundant under NT (Figs. 3C, 5 and 6). The dominant phylum was the Euryarchaeota, followed by the Thaumarchaeota and Crenarchaeota, all of which inhabit environments rich in organic matter. It remains to be determined if the greater abundance of Archaea in the NT system results from the higher soil organic-matter content, or if these microorganisms are more sensitive to CT-management practices. In any case, one important result from our study is that the Archaea are strong bioindicators of soil quality.

Differences in metagenomic composition were also attributed to crop management, but to a lesser extent than tillage practice. However, microorganisms that benefited from crop rotation were different in the NT and CT systems. Under NT, the abundance of Actinomycetales (Actinobacteria) was reduced under crop rotation (Fig. 7A), whereas with CT there was a decrease in seven other orders (Fig. 7B), with no clear pattern of associated functionality. In several previous studies from our group, we have been surprised by the lack of statistical differences between crop succession and rotation in parameters such as microbial biomass and activity and soil stocks of carbon and nitrogen (Balota et al., 1998; Franchini et al., 2007; Hungria et al., 2009; Silva et al., 2010, 2013). We might consider that the number of plant species in the rotation was not much higher than in the succession, with only a slight impact on microbial diversity. And certainly, we must keep in mind that the most important benefit of crop rotation is to break the plant–pathogen cycle.

Although our study has greatly improved our knowledge of microbial biodiversity in tropical-subtropical Oxisols, the high number of reads without any known hit is suprising and confirms that our understanding of the structure of soil microbial communities remains poor. Differences were attributed to the soil- and crop-management systems, and indications of microrganisms associated with higher sustainability were obtained. Our next step will be to explore the functionality of soil microorganims.

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