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COMPOSITION AND FUNCTION OF THE MICROBIAL COMMUNITY RELATED WITH THE NITROGEN CYCLING ON THE POTATO RHIZOSPHERE

Composición y función de la comunidad microbiana relacionada con el ciclaje de nitrógeno en la rizosfera de *Solanum tuberosum* (BAHUIN) grupo *phureja*

NATHALIA FLÓREZ-ZAPATA[1], M.Sc.; JUAN CARLOS GARCÍA[2], M.Sc; PATRICIA DEL PORTILLO[2], Microbiologa; SILVIA RESTREPO[3], Ph. D.; DANIEL URIBE-VELEZ[1], Ph. D.

[1] Laboratorio de Microbiología Agrícola, Instituto de Biotecnología, Universidad Nacional de Colombia. Avenida carrera 30 # 45-03. Bogotá D. C., Colombia

[2] Corporación Corpogen. Carrera 5 # 66A - 34. Bogotá D. C., Colombia

[3] Laboratorio de Micología y Fitopatología, Universidad de los Andes. Carrera 1 # 18 - 10. Bogotá, D. C., Colombia
Corresponding autor: Daniel Uribe Vélez, duribev@unal.edu.co

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ABSTRACT

In the *S. tuberosum* group *phureja* crops, mineral fertilizer and organic amendments are applied to meet the plants' nutritional demands, however the effect of such practices on the associated rizospheric microbial communities are still unknown. Nitrogen plays an important role in agricultural production, and a great diversity of microorganisms regulates its transformation in the soil, affecting its availability for the plant. The aim of this study was to assess the structure of microbial communities related with the N cycle of *S. tuberosum* group *phureja* rizospheric soil samples, with contrasting physical-chemical properties and fertilization strategy. Few significant differences between the community composition at the phylum level were found, only *Planctomycetes* phylum was different between samples of different soil type and fertilization strategy. However, the analysis of nitrogen-associated functional groups made by ribotyping characterization, grouped soils in terms of such variables in a similar way to the physical-chemical properties. Major differences between soil samples were typified by higher percentages of the ribotypes from nitrite oxidation, nitrogen fixation and denitrification on organic amendment soils. Our results suggest that, the dominant rhizosphere microbial composition is very similar between soils, possibly as a result of population's selection mediated by the rhizosphere effect. However, agricultural management practices in addition to edaphic properties of sampled areas, appear to affect some functional groups associated with the nitrogen cycling, due to differences found on soil's physical-chemical properties, like the concentration of ammonium that seems to have an effect regulating the distribution and activity of nitrogen related functional groups in the *S. tuberosum* rhizosphere.

Keywords: Nitrogen cycle; nitrification; nitrogenase; pyrosequencing; *Solanum phureja*

RESUMEN

Fertilización mineral y enmiendas orgánicas son aplicadas para satisfacer las demandas nutricionales de los cultivos de *S. tuberosum* grupo *phureja*. Sin embargo, el efecto de esas prácticas sobre la comunidad microbiana asociada a la rizósfera aún no se conocen. El nitrógeno juega un papel importante en la producción agrícola y una gran diversidad de microorganismos regulan su transformación en el suelo, afectando su disponibilidad para la planta. El objeto de este estudio fue determinar la composición de la comunidad microbiana de la rizósfera de *S. tuberosum* grupo *phureja*, asociada con el ciclo del nitrógeno, en muestras de suelo contrastantes en sus propiedades fisicoquímicas y estrategia de fertilización. Pocas diferencias significativas entre la composición de la comunidad microbiana a nivel de *phylum* fueron encontradas, únicamente el *phylum Planctomycetes* fue diferente entre las muestras de suelos con estrategias de fertilización diferentes. Sin embargo, el análisis de grupos funcionales asociados al nitrógeno llevado a cabo por la caracterización de ribotipificación, agrupó los suelos en términos de esas variables en una forma similar a las propiedades fisicoquímicas del suelo. Diferencias mayores entre las muestras de suelo fueron tipificadas por los altos porcentajes de ribotipos asociados a la oxidación de nitrito, fijación de nitrógeno y denitrificación sobre los suelos con enmiendas orgánicas. Nuestros resultados sugieren que la composición microbiana dominante es muy similar entre suelos, posiblemente como resultado de la selección de poblaciones mediada por el efecto rizosférico. Sin embargo, las prácticas del manejo agrícola en conjunto con las propiedades del suelo en las áreas muestreadas, parecen afectar algunos grupos funcionales asociados con el ciclo de nitrógeno, debido a las diferencias encontradas en las propiedades fisicoquímicas del suelo, como la concentración de amonio que parece tener un efecto regulando la distribución y actividad de los grupos funcionales relacionados con el ciclo del nitrógeno en la rizosfera de *S. tuberosum*.

Palabras clave: Ciclo del nitrógeno, nitrificación, nitrogenasa, pirosecuenciación, *Solanum phureja*.

INTRODUCTION

Potato is the fourth most important food crop worldwide, around of 324,2 millions of tons (MT) were produced in 2010, of which Colombia contributed with 2,1 MT for the same year, occupying the third place in terms of production in Latin America, (FAOSTAT, 2010). Colombia is also the major producer of *Solanum tuberosum* group *phureja* (Huaman and Spooner, 2002), with 150000 tons per year, making it a strategic crop due to its culinary recognition in international markets such as Europe and Japan (Porras, 1999; Rivera *et al.*, 2006). Besides, Colombia is part of the biodiversity center of this crop, which goes from the Peruvian mountains towards the north of the Andes in Colombia (Ghislain *et al.*, 2006; Rodríguez *et al.*, 2009).

Edaphic communities has become of great interest in recent years since it is well known that they contribute towards a broad spectrum of metabolic functions, stability, productivity and soil ecosystem resilience (Torsvik and Øvreas, 2002; Barrios, 2007; Brussaard *et al.*, 2007; Hajjar *et al.*, 2008). The edaphic metabolism of nitrogen (N), for instance, is carried out by a very intricate and diverse community which combines different transformations of this element due to its continuous interactions in the soil (Myrold, 2005; Hayatsu *et al.*, 2008). The understanding of the processes involved in nitrogen's availability in soil, is of great interest for agriculture, particularly for potato crops because even at high doses it may reduce tuber yield and quality (Giletto *et al.*, 2003).

In most of *S. tuberosum* group *phureja* crops, mineral fertilizers are applied to meet the plant's nutritional demands (Porras, 1999), however, in response to environmental problems that this practice entails such as increased nitrogenous gas emission (Galloway *et al.*, 2008), freshwater eutrophication (Smil, 1997;) and some negative effects on human health (McKenzie and Townsend, 2007), the application of organic amendments, such as manure, bokashi, crop residues and other renewable products, has been established.

Previous studies have shown differences in the composition of microbial communities, as a result of the rizospheric effect in relation to root location (Yang and Crowley, 2000); plant species (Smalla *et al.*, 2001; Haichar *et al.*, 2008) and plant cultivar (Briones *et al.*, 2002). In a similar way, different studies have demonstrated that factors such as agricultural practices (especially in relation with application of organic amendments), strongly affects soil's physical and chemical properties and disturbs the soil's microbial communities (Lundquist *et al.*, 1999; Bulluck *et al.*, 2002), so as the soil type does (Girvan *et al.*, 2003; Fierer and Jackson, 2006; Ge *et al.*, 2008).

On the other hand, recent culture-independent molecular techniques make it possible to explore microbial diversity more deeply and widely than ever before (Ge *et al.*, 2008), since it allows to overcome the technical limitations of culture-dependent methods (Nannipieri *et al.*, 2003; Kirk *et al.*, 2004; Roesch *et al.*, 2007). Within these methodologies, the bacterial tag-encoded FLX amplicon pyrosequencing method (Binladen *et al.*, 2007) is standing out. In this method, a selected variable region from the 16s rDNA gene is amplified from a DNA soil sample and then a unique tag for each individual sample is added to the amplicons. This step allows mixing amplicons from different samples into a single pyrosequencing run, reducing the sequencing cost per sample. Finally, in the downstream analyses, a huge number of DNA sequences are obtained and the tag is used to identify any given sequence related to the sample from which it was originated (Sun *et al.*, 2011). Recently, this method has been successfully applied to describe the soil's microbial communities across different sample locations (Roesch *et al.*, 2007; Lauber *et al.*, 2009), contrasting soil uses (Acosta-

Martínez *et al.*, 2008), evaluating the rhizosphere effect (Teixeira *et al.*, 2010; Uroz *et al.*, 2010) and in regards to N cycle studying the composition of ammonium-oxidizing (Roesch *et al.*, 2007; Uroz *et al.*, 2010) and N fixing bacteria communities in the soil (Uroz *et al.*, 2010).

Microbial community diversity of *S. tuberosum* group *phureja* rhizosphere soil ecosystem has been unexplored. Its study could give information about the microbial community related with the N cycle in this ecosystem. It may provide the bases to design better agricultural practices that promote the activity of specific groups that are able to increase the uptake of this nutrient by the plant, improving crop productivity. The aim of the present study was to analyze the edaphic communities structure related to different processes of N metabolism in rhizospheric soil samples from *S. tuberosum* group *phureja* crops, contrasting in terms of physical-chemical properties and fertilization strategy.

MATERIALS AND METHODS

Sampling Site Locations Description and Soil Samples Recollection

Spatially independent *S. tuberosum* group *phureja* rhizosphere soil samples were collected from four different crops, sowed with the same plant genotype (Clon 1), at flowering stage. These crops were located in the Cundinamarca department, one of the major *S. tuberosum* group *phureja* cropping areas in Colombia. Two crops, located at 4° 50' 60" N; 74° 16' 0" W and 4° 56' 0" N; 74° 10' 60" W, were mineral fertilized with 700 kg ha^{-1} of 14:30:15 (MF1) and 900 kg ha^{-1} of 12:24:12 (MF2) of NPK fertilizer application rates, respectively; similarly chemical compound inputs were applied for insect and pest control for both crops. Another two S. *phureja* crops, OA1 and OA2, which were neighbors to each other located at 5° 12' 0" N; 73° 52.60' 60" W had been organically fertilized with a bokashi like amendment (product of a composting process based on cattle manure, soil, molasses, yeast, rice husks and wood ash), for at least the last five years. These two crops were also characterized, because no chemical inputs for the control of pests and diseases had been applied. Soil's physical-chemical properties are presented in Table 1.

Rhizosphere soil were collected by shaking soil that were loosely adhering to the roots, from five plants randomly selected, after covered the crop area in zigzag, leaving 20-25 meters distance between sampled plants. Each sample was sifted by a 0,5 cm diameter hole sieve and then mixed in equal parts to obtain three subsamples per farm-land. Soil subsamples were stored at 4 °C for microbial and physical-chemical analysis of soil, and another portion of each soil subsample was stored at -20 °C for enzyme analysis and the last one was stored at -80 °C for the microbial soil community DNA analysis.

Physical-chemical analysis of soil was done at the Universidad Nacional de Colombia's Soils' Laboratory using standard methodologies for measuring: moisture, pH, organic carbon (OC), Ca, K, Mg, Na, cationic exchange capacity (CEC), P, Cu, Fe, Mn, B, Zn, clay, silt and sand percentage, total N (inorganic plus organic), and ammonium and nitrate mineral N.

Microbial Counts of Functional Groups Associated with the Edaphic Nitrogen Metabolism

Aerobic culturable nitrogen-fixing bacteria (NFB) were analyzed using plate count methodology after serial dilutions in modified NFb agar (Flórez-Zapata and Uribe-Vélez, 2011) and incubated at 28 °C for five days. Soil samples were analyzed in triplicate, and the morphotypes from each soil sample were isolated and cryopreserved at -80 °C.

Ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and denitrifier bacteria (DEN) abundance was determined by the most probable number (MPN) method (Pochon and Tardieux, 1962), using different carbon and N sources according to each group as follows: calcium carbonate (1 $g.L^{-1}$) was used as carbon source and ammonium sulphate (0,5 $g.L^{-1}$) and sodium nitrite (5 $g.L^{-1}$), as AOB and NOB N source, respectively. DEN were grown with sodium acetate (10 $g.L^{-1}$) and potassium nitrate (2 $g.L^{-1}$). Three dilutions by triplicate were inoculated with the soil sample dilution, and then incubated at 30 °C for 15 days for DEN and 30 days for AOB and NOB. After the incubation time, the presence/absence of each group was determined; from AOB nitrite production and for NOB nitrite consumption were determined with Griess-Ilosvay reagent (Alexander and Clark, 1965); for estimating DEN, nitrite consumption and gas production were determined by using Durham's bells (Horiba *et al.*, 2005). Microorganism MPN was estimated by using McCrady's tables (1918); and counts were expressed as MPN of microorganisms by gram of dry weight soil.

Table 1. Physical-chemical characteristics of the soil samples being studied.

Soil Sample	Applied Amendment Nature	Texture	% M	pH	OC	K	P	N	NH_4	NO_3
MF1	Mineral	Loam	79.10	5.6	14.5	1.44	11.7	1.08	24.5	43.0
MF2	Mineral	Loam	52.81	5.5	12.9	1.8	73.7	0.59	31.2	12.7
OA1	Organic	Sandy Loam	80.01	5.7	13.3	2.25	69.8	0.93	11.1	15.5
OA2	Organic	Sandy Loam	62.33	6.0	9.74	1.64	49.8	0.62	11.7	7.94

% M, percentage of moisture. OC, percentage of organic carbon, K, potasium (mmol· 100 g^{-1}), P, phosphorus (mg · Kg^{-1}), N, percentage of total nitrogen, NH_4, ammonium (mg · Kg^{-1}) and NO_3, nitrate (mg · Kg^{-1}).

Soil Microbial Community DNA Extraction and Bacterial Tag-Encoded FLX Amplicon Pyrosequencing Analysis

Soil DNA was extracted using a Power Soil kit TM DNA (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. For bacterial tag-encoded FLX amplicon pyrosequencing analysis (referred as ribotyping analysis), PCR reactions for the 16S rDNA V5 and V6 variable regions were performed using the primers reported by Sogin *et al.*, (2006): 807F (5'-GGATTAGATACCCBRGTAGTC-3') and 1050R (5' AYTGDCGACRRCCRTGC-3'). The PCR reaction was carried out in 25 µL containing 2,5U of Pfu Turbo polymerase (Stratagene, La Jolla, CA, USA), 1X reaction buffer, 0,6 mM dNTPs, 0,75 µM of each primer, 5 % v/v DMSO and 2-8 ng DNA. PCR conditions for 16S rDNA amplifications started with an initial denaturing step at 95 °C for two min, followed by 30 cycles of 95 °C for 1 min. A touch-down step at 60 °C to 51 °C (0,33 °C/cycle) and extension temperature step at 72 °C for one min were followed by a final extension step at 72 °C for five min.

A 1:10 dilution of the PCR product was used as template for a second PCR to add the pyrosequencing primers that contains the adapters and the respective barcode specific for each soil analyzed. The reaction was carried out using the same conditions described above, except for the annealing temperature and the number of cycles (five cycles at 53 °C). PCR product quality was assessed on 1 % w/v agarose gel. Once confirmed, PCR products were cleaned using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA), following the manufacturer's instructions. Samples were sent to the Environmental Genomics Core Facility at the University of South Carolina (Columbia, SC, USA) for pyrosequencing on a Roche Diagnostics 454 Life Sciences Genome Sequencer FLX (Indianapolis, IN, USA) machine.

Sequence Quality Trimming

The pyrosequencing sequences obtained were sorted into specific samples according to the specified barcode. Sequences lacking tags were discarded. Once each DNA set of sequences had been obtained, 454 sequencing primers and barcodes were cleaned. Then, less than 50 nucleotide length DNA sequences, low quality regions with Q-values less than 20 and with one or more ambiguous base (N) were eliminated from the analysis to minimize any bias due to random sequencing errors. Each ribotype's taxonomic identity was performed at genus level using the Classifier tool (with default parameters) from the Ribosomal Database Project (Wang *et al.*, 2007), available on the website from the Ribosomal Database Project.

Ribotype's Assignation into Nitrogen Edaphic Metabolism Process

A database was constructed through an extensive bibliographic search of the different genera associated with nitrogen-fixation, nitrification, denitrification and dessimilative reduction of nitrate to ammonium processes (Tiedje, 1988; Young, 1992; Teske *et al.*, 1994, Malik *et al.*, 1997; Chelius and Triplett, 2000; Chen *et al.*, 2003; Behar *et al.*, 2005; Myrold, 2005; Zakhia *et al.*, 2006). This search led to identifying around 140 genera associated with all these processes (Table S1). The four soil sample ribotypes genera assignation, were compared with our database to identify which genera were present and potentially forming part of a particular functional group (functional ribotyping analysis). Each estimated population's percentage was estimated in relation to total DNA sequences from a soil sample to provide a cumulative percentage for each functional group.

Determination of Soil's Nitrogenase Enzymatic Activity

The soil's nitrogenase activity was determined by the acetylene reduction test, according to the methodology suggested by Hardy *et al.*, (1968), with some modifications. One mililiter of soil sample dilution at 10-3 and 10-4 was inoculated by triplicate into amber bottles containing 9 mL of the semisolid modified NFb Agar (Flórez-Zapata and Uribe-Vélez, 2011). The samples were incubated for 24 hours at 28 °C. Then 10 % of the bottles' atmosphere was replaced by acetylene and incubated again as before. A Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, USA), was used for determining ethylene production which was reported as nmoles of ethylene per gram dry weight of soil during 24 hours.

Sequencing and Analysis of 16s rDNA From Nitrogen-Fixing Bacteria

Total DNA from each NFB morphotype was isolated by the CTAB phenol-chloroform method (Ausubel *et al.*, 2003). The 16S rDNA gene was amplified by using universal primers 27f and 1492r under standard conditions (Martin-Laurent *et al.*, 2001). PCR products were sequenced in both directions by Macrogen Inc, Korea using the ABI 3730xl DNA analyzer sequencer. Then, obtained sequences were analyzed by the SeqMatch tool from the Ribosomal Database Project (Cole *et al.*, 2009) to obtain taxonomic assignation.

Statistical Analysis

Microbe counts were expressed in logarithms and significant statistically differences were verified by using the Kruskal-Wallis (KW) test by pairs at p=0,05 significance level (Kruskal and Wallis, 1952). Nitrogenase activity was analyzed by Tukey test at the same significant level. A Z test proportions analysis was performed, to determine whether differences at phylum level in the percentage of microorganisms obtained by pyrosequencing were statistically significant (p=0,05 significance value). A hierarchical cluster analysis was done using each soil's physical-chemical and biological variables (functional ribotyping analysis), according to average linkage clustering method, STATA 9.0 (StataCorp, 2005) was used for all statistical analysis.

RESULTS

Soil Physical-Chemical Analysis

Table 1 presents the soil samples' physical-chemical characteristics. Some differences were found in the soils in terms of organic carbon, total N and soil moisture content, independently of sampling location or fertilization strategy. However, it was observed that OM1 and OM2 soils had slightly higher pH values and smaller ammonium concentrations probably due to the fertilization strategy of these soils. Also, the topsoil texture was different between soils, for instance soils MF1 and MF2 were classified as loam, while OM1 and OM2 were classified as sandy loam.

A hierarchical cluster analysis done with the physical-chemical variables grouped the analyzed soils into two clusters, with MF1 and MF2 in one cluster and soils OA1 and OA2 in the other cluster (Fig.1). Such clustering indicates that despite the fact all soils are Andisols with a high organic content (above 16 %), both, soils' structure and fertilization strategy seems to have a strong effect on their physical-chemical variables in such a way that allowed the clustering into two different groups of soils.

Abundance of Culturable Microbial Functional Groups Associated with Edaphic Nitrogen Metabolism

Some differences were observed between the soils being studied regarding the biological variables associated with the nitrogen cycle (enzyme activity and microbial counts). The microbial counts for MF1 and MF2 presented the highest values for all functional groups analyzed, except for AOB in farm MF1 (Table 2). Even AOB for farm MF2, presented counts two orders of magnitude above the other soils. On the other hand, despites the higher numbers of NFB counts in mineral fertilized soils, nitrogenase activity was higher on soils OA1 and OA2.

Culture Independent Analysis of Soil Microbial Community at Phylum Level

After the sequence trimming process, a total of 24423 partial 16S rDNA sequences were obtained from the four soil samples. The phylum composition was determined from Bacteria and Archea domain, the proportions for each phylum for the four soil samples analyzed are presented in the Table 3. Small differences in the proportions of the phyla were found between soils, in fact only Plactomycetes showed significant differences between mineral fertilized and organic amended soils. The most prevalent bacteria phyla in all the soil samples were *Proteobacteria*, Bacteroidetes, Plactomycetes, Actinobacteria, *Verrucomicrobia* and *Acidobacteria*, while *Crenarchaeota* was the most prevalent for the Archea domain. Besides, *Spirochaetes* and *Deinococcus-Thermus* phyla were only found in OA1 and OA2 soils.

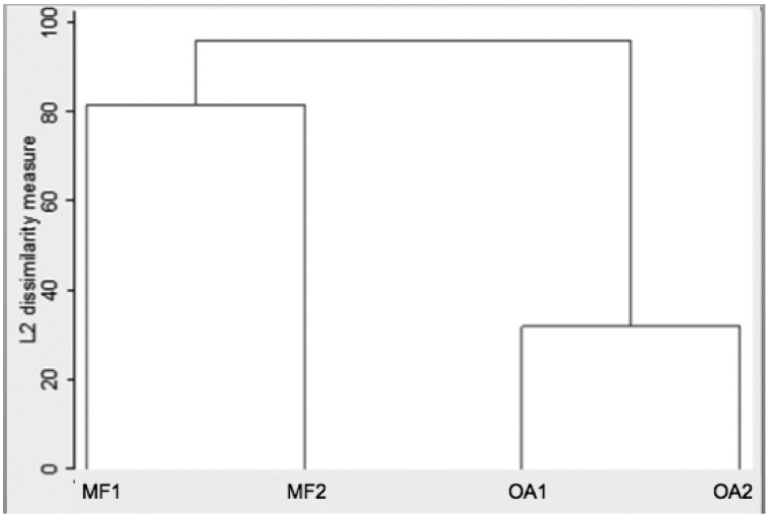

Figure 1: Hierarchical cluster analysis of the physical-chemical characteristics of the soils being studied

Table 2. Microbial counts and nitrogenase activity for some functional groups associated with nitrogen metabolism.

	MF1	MF2	OA1	OA2
Ammonium Oxiding Bacteria*	1.15×10^2 a	1.02×10^4 c	6.99×10^2 ab	2.00×10^2 b
Nitrite Oxiding Bacteria*	1.58×10^4 a	8.90×10^4 c	1.79×10^3 b	6.98×10^3 abc
Denitrifiers*	$> 2.51 \times 10^6$	$> 2.14 \times 10^6$	2.44×10^2	1.33×10^6
Nitrogen Fixing Bacteria**	2.16×10^8 b	2.79×10^8 a	6.03×10^7 d	9.74×10^7 c
Nitrogenase Activity***	2787.17 b	3139.35 b	14877.77 a	4286.52 b

* MPN g-1 dry weight soil. ** CFU g-1 dry weight soil. *** nmoles of ethylene per gram of dry weight soil in 24 hours.

Table 3. Sampled soil microbial composition at the phylum level. Values correspond to the proportion of the good quality sequences of each phylum in relation to the total obtained for each farm. Values having the same letter were not statistically significantly different, as described by Z test for proportions (p = 0,05).

	MF1	MF2	OA1	OA2
Archaea Domain	2.10 c	6.59 a	2.05 c	4.88 b
Phylum Euryarcheota	0.05 b	0.11 b	0.03 b	0.32 a
Phylum Crenarchaeota	1.50 b	3.72 a	1.28 b	3.34 a
Unclassified Archaea Domain	0.54 ac	2.76 a	0.74 bc	1.22 b
Bacteria Domain	91.47 b	87.23 c	93.26 a	90.73 b
Phylum Cyanobacteria	0.00 a	0.00 a	0.00 a	0.02 a
Phylum OD1	0.03 a	0.01 a	0.06 a	0.05 a
Phylum Chlamydiae	0.11 a	0.09 a	0.13 a	0.15 a
Phylum WS3	0.12 ab	0.03 a	0.03 b	0.00 ab
Phylum Firmicutes	0.45 a	0.23 ab	0.19 ab	0.15 b
Phylum OP10	0.12 a	0.03 a	0.13 a	0.02 a
Phylum Bacteroidetes	2.45 b	2.08 c	6.64 a	2.96 b
Phylum Planctomycetes	2.27 c	3.45 b	4.75 a	5.13 a
Phylum Actinobacteria	6.48 a	3.81 b	3.21 b	3.89 b
Phylum Chloroflexi	0.49 a	0.06 b	0.58 a	0.62 a
Phylum Nitrospira	0.11 a	0.07 a	0.10 a	0.17 a
Phylum Verrucomicrobia	4.30 a	3.87 ab	3.82 ab	3.39 b
Phylum Gammatimonadetes	0.34 a	0.10 b	0.16 ab	0.10 b
Phylum TM7	1.10 b	1.35 ab	1.70 a	1.40 ab
Phylum Proteobacteria	29.73 bc	28.15 c	37.52 a	29.97 b
Phylum Acidobacteria	5.60 a	3.95 bc	3.18 c	4.01 b
Phylum Spirochaetes	0.00 a	0.00 a	0.03 a	0.05 a
Phylum Deinococcus-Thermus	0.00 a	0.00 a	0.03 a	0.05 a
Phylum Thermotogae	0.00 a	0.00 a	0.06 a	0.00 ab
Unclassified Bacteria Domain	37.77 b	39.94 a	30.94 c	38.59 ab
Unclassified Domain Sequences	6.43 a	6.19 a	4.69 b	5.08 b

Nitrogen Cycle Functional Groups Ribotypes Association

The genera of the microorganisms present in the soil samples being studied were associated with different N cycle functional groups previously identified through literature reports such as nitrogen-fixing bacteria, nitrifiers, denitrifiers and bacteria that can dissimilate nitrate to ammonium (DRNA). We would point out that the presence of a particular genus does not assure its active role in N transformation in the soil. Nevertheless, this information can be interpreted as an approach for determining the functional potential of a soil being studied, to transform a particular element and its relationship with other N cycle-related variables, especially into the N cycle which is mainly driven by the bacteria and archea domains.

The first process of the cycle analyzed was nitrification, through ammonium oxidation and nitrite oxidation. Only *Nitrosospira* ribotypes (16S rDNA sequences), were found to fulfill this function for AOB, and sequences from the phylum *Crenarchaeota* for the ammonia-oxidizing *archaea* (AOA) (Fig. 2A). The genus Nitrospira and Nitrobacter were identified regarding nitrite oxidation, but Nitrobacter genus was only found on OA1 and OA2 soils, suggesting some differences as a result of sampling location (soil type) or fertilization strategy. The results obtained by MPN for the ammonium oxidation group (Table 2) did not show equivalent distributions with the information obtained through ribotyping analysis (Fig. 2A). Likewise, the nitrite oxidizer group had an opposing trend because the ribotypes potentially related to this group indicated a higher proportion on OA1 and OA2 soils, contrary to MPN results.

Other processes related to nitrate reduction were also considered, such as denitrification and dissimilative reduction of nitrate to ammonium (DRNA) (Fig. 2C-D). Opposite to MPN technique, higher numbers of denitrification-related sequences were observed on OA1 and OA2 (Fig. 2C; Table 2). Regarding organisms taking part in DRNA, soils of farm OA1 (followed by MF1) had the highest proportion of sequences for this group regarding the total amount of sequences obtained for each farm, while soils MF2 and OA2 had similar proportions of such sequences in their soil communities.

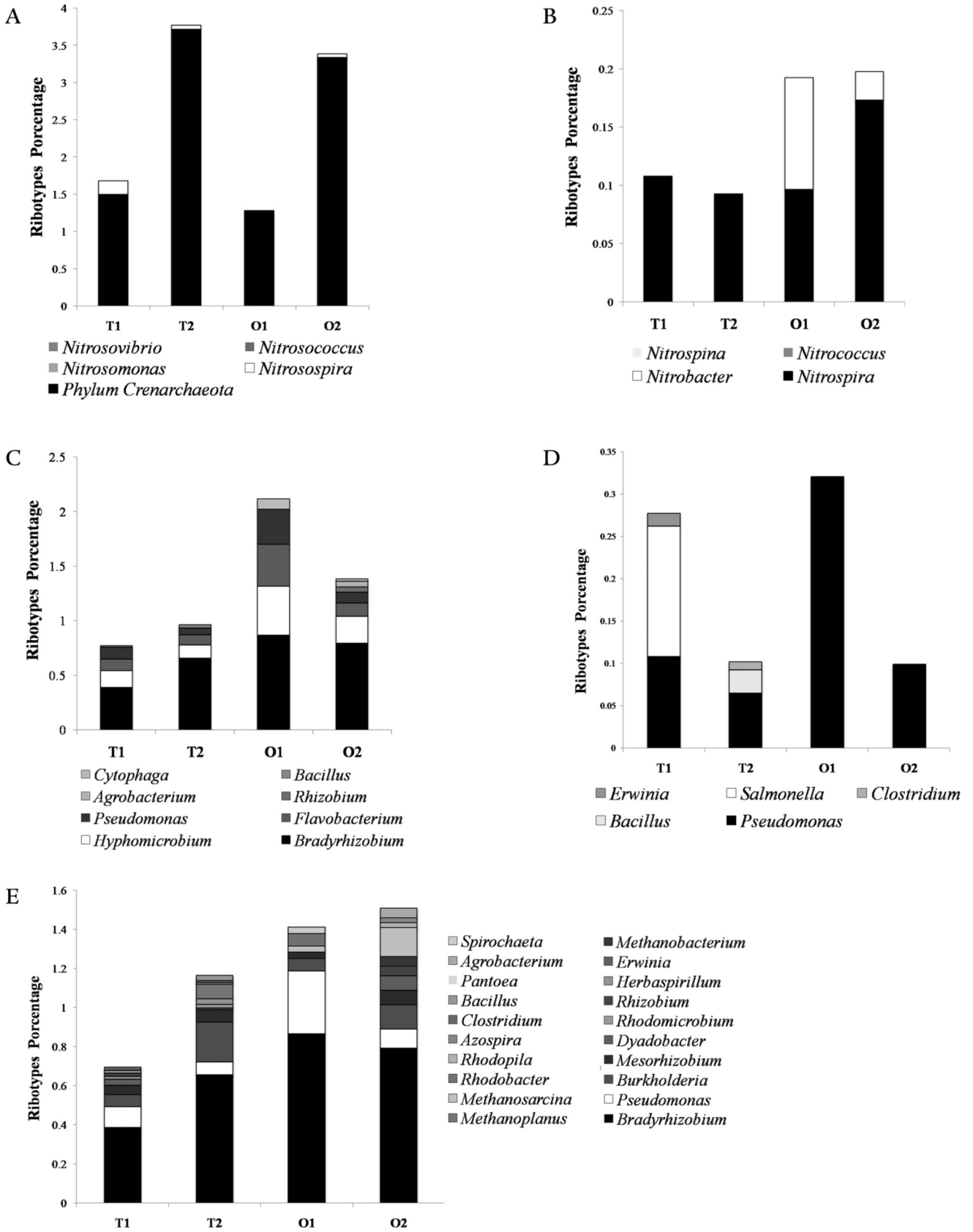

Figure 2: Proportion of ribotypes associated with the different steps related with the nitrogen cycle: A: Ammonium oxidation. B: Nitrate oxidation. C: Denitrification. D: Dissimilative reduction of nitrate to ammonium. E: Biological nitrogen fixation.

Lastly in terms of NFB (Fig. 2E), soils MF2 and OA2 presented the highest numbers of genera associated, with 11 for each of them and farm OA1 presented seven genera, which represents the lowest number. Despite the differences in the number of genera described above, a trend appeared where the OA1 and OA2 soils showed the higher proportions of sequences associated with this group (Fig. 2 E). This result was contrary to the trend found in the plate count approach, but similar to the nitrogenase activity found in the soil samples (Table 2).

A hierarchical cluster analysis was done using the ribotyping description of community structure in terms of the cumulative proportion of each nitrogen-associated genus per farm. This analysis showed a clear separation of the proportion of ribotypes from soils samples in a similar fashion to the physical-chemical characteristics of soils clustering (Fig. 1) with MF1 and MF2 in one cluster and soils OA1 and OA2 in the other cluster (results not shown).

Culturable Nitrogen-Fixing Bacteria 16s rDNA Sequence Analysis

16S rDNA sequences from culturable NFB isolates obtained from the plate count, were compared with our database and with the genera identified in the ribotyping analysis. Thirteen genera were identified out of 21 strains isolated from the four soils, of which *Microbacterium*, *Serratia*, *Enterobacter* and *Pseudomonas* had the highest frequency (4, 3, 4 and 2 strains assigned, respectively). The remaining genera were only represented by one isolate. Figure 3, shows the comparison of relative abundance of nitrogen-fixing ribotypes assigned to isolates obtained on agar NFb plate count (Fig. 3A) and from high throughput sequencing of 16 S rDNA gene variable regions (V5-V6) from four farms (Fig. 3B).

As expected, a higher number of genera with the potential to carry out such function were found in the soil samples by pyrosequencing analysis than by morphotype selection in NFb medium. However, the genus found in the culture dependent methodology for example for farm MF1, such as *Serratia*, *Microbacterium* and *Cellulomonas*, were not found in the information obtained by pyrosequencing (Fig. 3). Similarly, some genera, such as *Bradyrhizobium*, *Mesorhizobium* and *Burkholderia*, were relatively highly represented by high throughput sequencing ribotyping results, but were not found by culture dependent technique. Similar results were obtained with soils MF2, OA1 and OA2 (Fig. 3).

DISCUSSION

The aim of this study was to search any relationship between soil physical-chemical environment and the microbial community associated with the nitrogen cycle, which is mainly driven by the prokaryotic soil community. The hierarchical cluster analysis of the soils, allowed separating the mineral and organically fertilized farms in two clusters in relation with its physical-chemical characteristics. Such clustering indicates that either, physical-chemical background of the soil, influenced by environmental and edaphic factors or fertilization management or even the interaction between those two variables have a strong effect to differentiate soils in terms of physical-chemical parameters.

Without neglecting the importance of soil origin or fertilization strategy in defining soil clusters, our results strongly indicates that structure and function of soil microbial community associated with N cycle is mainly affected by the soil physical-chemical environment. Similar results were found by Rasche *et al.*, (2006), who in a microcosm assay found a stronger effect of soil type on the microbial community, when compared with different potato genotypes.

It is important to mention that the four soils analyzed in this study have high organic matter (OM) content, due to their location on high mountains of the Andes, which are characterized by low temperatures and mineralization rates. Therefore, organic amendments application on the organically fertilized soils did not resulted in higher OM levels, as observed on soils OA1 and OA2 which showed similar OM levels when compared with mineral fertilized soils (MF1 and MF2). On contrary, the strongest differences in both clusters of soils in terms of the physical-chemical variables were defined by the ammonium content. Higher levels of ammonium were found in MF1 and MF2 soils, as a consequence of the high inorganic fertilization (100-150 kg ha^{-1}), compared with OA1 and OA2 soils, where the N applied is from organic sources only. It is well known that ammonium is directly regulating different process within the N cycle such as, N fixation, nitrification, anaerobic ammonium oxidation and nitrate assimilation (Rice and Tiedje, 1989; Robertson, 1989; Strous *et al.*, 1999; Dixon and Kahn, 2004; Booth *et al.*, 2005; Myrold, 2005). Therefore, differences in the proportion of ammonium, may explain part of the divergence in microbial populations associated with N metabolism in soils with different fertilization strategies.

Bacterial phyla composition analysis in the four contrasting soils showed nineteen different phyla. The most abundant phyla observed in this study (*Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Bacteroidetes* and *Verrucomicrobia*), has been previously reported by other authors as being prevalent in agricultural and nonagricultural soils (Janssen *et al.*, 2006; Roesch *et al.*, 2007; Acosta-Martínez *et al.*, 2008; Urich *et al.*, 2008; da Rocha *et al.*, 2009). This result supports the observations of Fulthorpe *et al.* (2008), who indicate that the same major phyla are always present in a particular soil type, regardless of soil location. However, some phyla distribution in our study had a peculiar pattern, suggesting that the community structure, at the phylum level, can be site specific (Fulthorpe *et al.*, 2008; Teixeira *et al.*, 2010).

The proportion of representative phyla observed in this work was different regarding previous studies. For instance, the phylum *Acidobacteria* have been found highly represented (above 20 %) in other studies (Jansen et al 2006; Lee *et al.*,

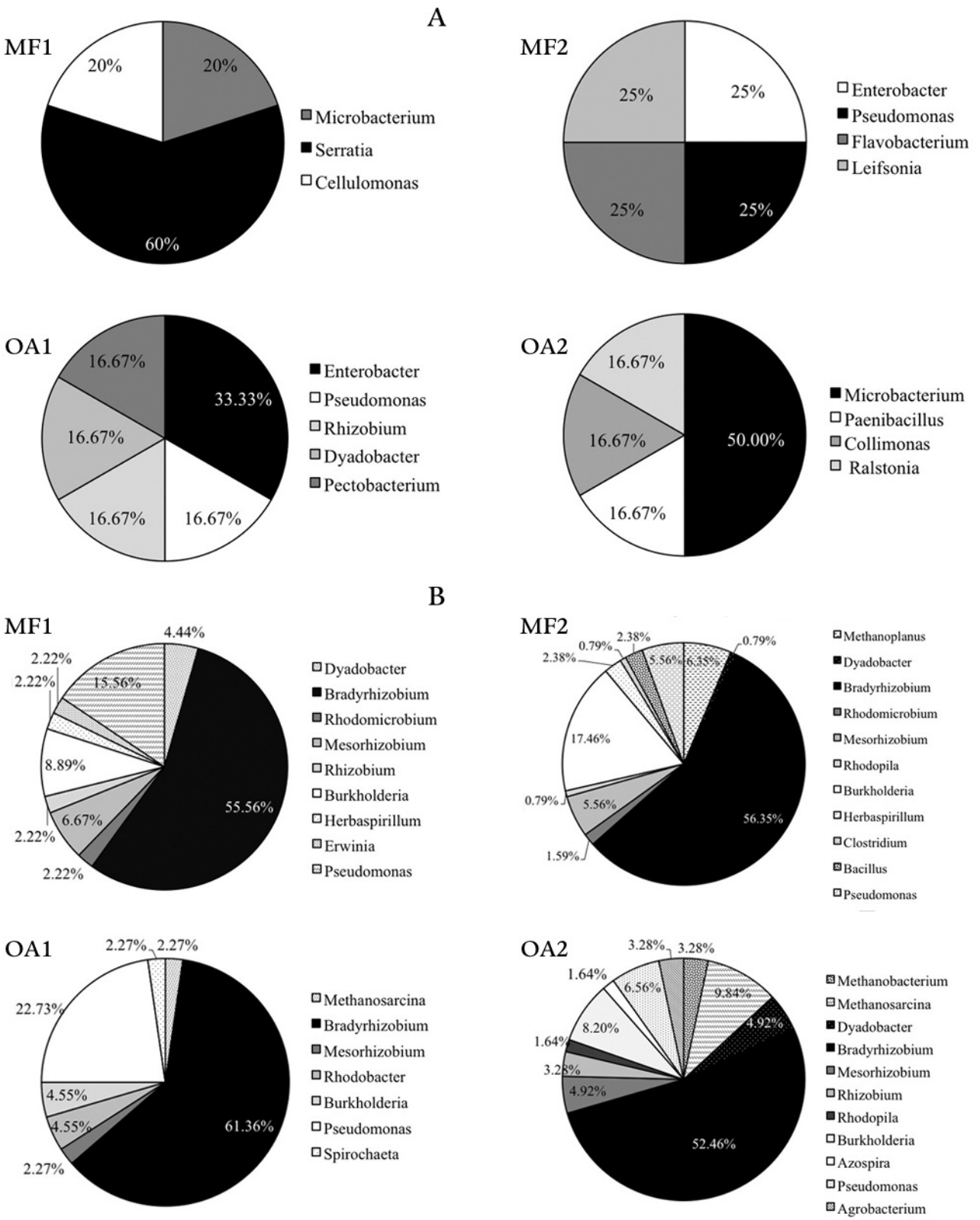

Figure 3: Relative abundance of nitrogen-fixing bacteria assigned by 16s rDNA sequencing of NFb isolates (A) and ribotyping sequence analysis (B) obtained from MF1, MF2, OA1 and OA2 potato rhizosphere soil samples.

2008; Jones *et al.*, 2009), indicating that organisms from this phylum are dominant in rhizosphere soils. However, the results obtained in this study, indicated that such group only represents 6 % of total sequences from the *S. tuberosum* group *phureja* rhizosphere soil. This finding may be a result of the high OM content of the soils samples analyzed here, a condition apparently limiting *Acidobacteria* development, as these bacteria are oligotrophic microorganisms that seem to have negative correlations with soil carbon content (Lee *et al.*, 2008, Jones *et al.*, 2009).

The phylum *Proteobacteria*, which was the most abundant in the soils being studied (28 %-37 %), has often been reported as being the most commonly found in soil (Filion *et al.*, 2004, Janssen *et al.*, 2006; Spain *et al.*, 2009). This result corresponded with the observation that this group of organisms is both genetically-diverse and metabolically-versatile (Lee *et al.*, 2008), a condition allowing it to be highly competitive after the presence of a disturbing event in the environment. On the other hand, the phylum *Bacteroidetes* has been reported in high proportions in agricultural soils characterized by chemical input application regarding undisturbed soils (forest or forest reserves) (Roesch *et al.*, 2007; Acosta-Martínez *et al.*, 2008). Nevertheless, such group showed a tendency of being present in higher proportions in organically amended soils in the present study. Our results showed also the phylum *Verrucomicrobia* as one of the most abundant in the rhizosphere of the *S. tuberosum* group *phureja*. Similar results have been recently reported by da Rocha *et al.*, (2010), who found that such phylum numerically dominates the communities of the *S. tuberosum* rhizosphere, suggesting that *Verrucomicrobia* may have an important role in that ecological niche

Few significant differences in phyla proportions were found between the two sources of soil samples, *Planctomycetes* phylum, was the only one appearing in significantly different proportions between mineral fertilized and organic amended soil management (greater proportions in the latter). This result is in agreement with the observation of Girvan *et al.*, (2003), who in spatially different soil samples with different management practices, found almost identical bacterial profiles between samples, suggesting that despite heterogeneity in soil properties, the dominant members of the microbial community appeared to be ubiquitous. In the same way, Martiny *et al.*, (2006) discussed that microbial biogeographic distribution are taxonomic-scale dependent, as differences in patterns present at finer taxonomic resolutions, might not be reflected at broader resolutions, as is the case for the phylum taxonomic level of this study. The importance of differences in *Planctomycetes* phylum, should be studied in more detail since it has been suggested that organisms from this phylum play an important role in the N cycle through the anaerobic ammonium oxidation function, known as anammox (Wagner and Horn, 2006; Elshahed *et al.*, 2007). In this sense, Uroz *et al.*, (2010) found that this phylum have greater representation than aerobic AOB in the soil of an oak forest, suggesting that, the bacterial community has the ability to mobilize an alternative mechanism (anammox) for the oxidation of ammonium. The high proportion of such phylum found in the organically fertilized soils under study suggests that a similar situation may be happening in those soils.

The analysis of ammonia oxidation-associated Eubacterial ribotypes showed only representatives from the genus *Nitrosospira* sp. (Fig. 2.A). This result agreed with other studies (Belser, 1979; Bothe *et al.*, 2000) that highlighting the genus *Nitrosospira* sp. as being the most widely AOB microorganism in soil. On the other hand, the Archaea phylum *Crenarchaeota* was also present in high proportions in the evaluated soils, particularly on soils OA2 and MF2. This is important because *Crenarchaeota* has been recently proposed as playing a major role in ammonium oxidation in soil due to its high proportion and distribution found in both pristine and agricultural soils from three climatic zones (Leininger *et al.*, 2006; Nicol and Schelp, 2006; Urich *et al.*, 2008).

Analysis of the sequences potentially related to nitrite oxidation showed that organically managed soils had nearly two-fold more ribotypes in terms of the percentage of total population regarding mineral fertilized soils. The genus Nitrobacter was only found on organic amended soils, suggesting an effect of soil management on this functional group's distribution. It has been described that higher ammonium concentration, a typical situation on soils having mineral fertilization, may inhibit nitrifier population (Belser, 1979). Besides, higher pH values, like those found in the organically managed soils being studied, especially in the OA2 farm, positively affect this functional group's population (Myrold, 2005). All the above evidence may explain the higher proportions of ribotypes associated with nitrite oxidizer microorganisms found in organically-managed soils in this study.

The denitrification-associated ribotypes tended to have a higher proportion of microorganisms and an even higher number of genera associated with this process in organically-amended soils. Kramer *et al.*, (2006) not only observed higher numbers of denitrifier bacteria in a similar study of N dynamics in soils fertilized with organic amendments, but also its relation with lower nitrate amounts and increased nitrifying microorganism activity, as found in our study. These authors indicated that such findings are important because increased soil denitrifying activity leads to higher nitrate consumption, thereby reducing the amount of this compound being leached and therefore reducing the adverse environmental effect associated with this phenomenon. The high correlation between nitrogen-fixing bacteria, estimated by culture-independent approaches and soil enzymatic activity, indicated the importance of use robust culture independent techniques, such as pyrosequencing, to establish relationships between functionality and community composition in a soil ecosystem. On the other hand, there was no correspondence between the taxonomic allocation of microorganisms isolated from culture media and ribotyping sequences obtained in the soils.

The above results show one of the main limitations of culture dependent techniques, which incorporate bias related to growth medium factors, such as high carbon source concentration. Organisms usually under-represented in the environment (i.e. Enterobacteriaceae) may thus be able to growth rapidly in laboratory conditions, providing a distorted view of their distribution in the environment (National Research Council (US), 2007). However, it should be stressed that both techniques are complementary since the microorganisms that are not described by the ribotyping approach, were detected from 16S rDNA gene sequencing of cultured microorganisms. Besides, as annotated by Soares *et al.*, (2006), cultivable approaches have the added advantage of microorganism isolation which allows studying their physiology and bio-technological potential.

CONCLUSIONS

This study provided a starting point for assessing the microbial community's diversity and functionality in *S. tuberosum* group *phureja* rhizosphere and provides additional information about the factors that affect the soil microbial biodiversity distribution, associated with the N cycle. The results showed a grouping similar to that seen with the physical-chemical properties of soil, when making global cluster analysis of variables associated with N metabolism obtained by high-throughput culture independent methods. This result in addition with the others discussed above, allowed us to conclude that both factors, soil type and fertilization strategy, by themselves or combined, have an effect on edaphic physical-chemical properties that in turn determine the microenvironments, where soil microorganisms thrive, thereby affecting their distribution and functional activity in an ecosystem.

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Supplementary material
Table S1. Microbial genera related with nitrogen cycle

1. Ammoniun Oxidation
Nitrosomonas
Nitrosospira
Nitrosococcus
Nitrosovibrio
Crenarchaeota

2. Nitrite Oxidation
Nitrobacter
Nitrococcus
Nitrospina
Nitrospira

3. Nitrogen Fixation
Halobacterium
Methanobacterium
Methanothermus
Methanococcus
Methanoplanus
Methanosarcina
Methanolobus
Cyanothece
Gloeocapsa
Anabaena
Plectonema
Lyngbya
Spirulina
Gloeothece
Nostoc
Calothrix
Oscillatoria
Chloroherpeton
Prosthecochloris
Chlorobium
Pelodictyon
Frankia
Arthrobacter
Propionibacterium
Streptomyces
Beijerinckia
Methylosinus
Rhodopseudomonas
Photorhizobium
Bradyrhizobium
Mycoplana
Rhodomicrobium
Mesorhizobium
Rhizobium
Sinorhizobium
Xanthobacter
Ancylobacter
Rhodobacter
Gluconacetobacter
Rhodopila
Azospirillum
Rhodospirillum
Derxia
Burkholderia
Herbaspirillum
Thiobacillus
Aquaspirillum
Azoarcus
Azospira
Rhodocyclus
Alcaligenes
Desulfobacter
Desulfovibrio
Paenibacillus
Clostridium
Heliobacillus
Heliobacterium
Heliospira
Desulfotomaculum
Propionispira
Bacillus
Acidithiobacillus
Chromatium
Thiocapsa
Chromatium
Thiocystis
Ectothiorhodospira
Klebsiella
Enterobacter
Escherichia
Erwinia
Pantoea
Methylobacter
Methylococcus
Methylomonas
Pseudomonas
Azotobacter
Azotococcus
Beggiatoa
Vibrio
Citrobacter
Synechococcus
Synechocystis
Chroococcidiopsis
Dermocarpa
Myxosarcina
Pleurocapsa
Xenococcus
Pseudoanabaena
Aphanizomenon
Cylindrospermum
Nodularia
Scytonema
Chlorogloeopsis
Fischerella
Geitlerinema
Stigonema
Prochloron
Campylobacter
Acetobacter
Agrobacterium
Azorhizobium
Chromobacterium
Methylocystis
Desulfosporosinus
Phormidium
Serratia
Spirochaeta
Treponema
Desulfomicrobium
Methanothermobacter.
Methanopyrus
Tolypothrix
Wolinella
Methanobrevibacter
Desulfonema
Acetobacterium

3. Denitrification
Alcaligenes
Agrobacterium
Aquaspirillum
Azospirillum
Bacillus
Blastobacter
Bradyrhizobium
Branhamella
Chromobacterium
Cytophaga
Flavobacterium
Flexibacter
Halobacterium
Hyphomicrobium
Kingella
Neisseria
Paracoccus
Propionibacterium
Pseudomonas
Rhizobium
Wolinella
Rhodopseudomonas
Nitrosomonas
Thiobacillus
Thiomicrospira

4. Desassimilative Reduction of Nitrate to Ammonium
Clostridium
Desulfovibrio
Selenomonas
Veillonella
Wolinella
Citrobacter
Enterobacter
Erwinia
Escherichia
Klebsiella
Photobacterium
Salmonella
Serratia
Vibrio
Campylobacter
Bacillus
Neisseria
Pseudomonas