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Bacterial communities in the rhizosphere of *Vitis vinifera* L. cultivated under distinct agricultural practices in Argentina

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Abstract Plants interact with a myriad of microbial cells in the rhizosphere, an environment that is considered to be important for plant development. However, the differential structuring of rhizosphere microbial communities due to plant cultivation under differential agricultural practices remains to be described for most plant species. Here we describe the rhizosphere microbiome of grapevine cultivated under conventional and organic practices, using a combination of cultivation-independent approaches. The quantification of bacterial 16S rRNA and *nif*H genes, by quantitative PCR (qPCR), revealed similar amounts of these genes in the rhizosphere in both vineyards. PCR-DGGE was used to detect differences in the structure of bacterial communities, including both the complete whole communities and specific fractions, such as *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, and those harboring the nitrogen-fixing related gene *nif*H. When analyzed by a multivariate approach (redundancy analysis), the shifts observed in the bacterial communities were poorly explained by variations in the physical and chemical characteristics of the rhizosphere. These approaches were complemented by high-throughput sequencing (67,830 sequences) based on the V6 region of the 16S rRNA gene, identifying the major bacterial groups present in the rhizosphere of grapevines: *Proteobacteria*, *Actinobacteria*, *Firmicutes*,

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Bacteriodetes, *Acidobacteria*, *Cloroflexi*, *Verrucomicrobia* and *Planctomycetes*, which occur in distinct proportions in the rhizosphere from each vineyard. The differences might be related to the selection of plant metabolism upon distinct reservoirs of microbial cells found in each vineyard. The results fill a gap in the knowledge of the rhizosphere of grapevines and also show distinctions in these bacterial communities due to agricultural practices.

Keywords Bacterial communities · Cultivation-independent analysis · Plant–microbe interactions · Rhizosphere microbiome · Organic management

Introduction

Argentina is the fifth-largest producer of wine in the world, ranking immediately after traditional wine-producing countries such as Australia, Spain, Italy and France. However, the export boom in Argentinian wines is very recent (Farinelli 2007) and is today the principal destination of the grapes (*Vitis vinifera* L.) cultivated in Argentina. The west-central region of the country (provinces of San Juan and Mendoza) accounts for 92 % of the national vineyard crop area, where grapes are cultivated under distinct production systems and management, according to the availability of water (commonly provided by human intervention); (Abraham et al. 2009). In the year 2011, production was approximately 785,000 metric tons, of which 90 % was used for the production of wine (Instituto nacional de Vitivinicultura 2011).

The productivity of agricultural systems is greatly dependent on the functional processes of soil microbial communities, more specifically the rhizosphere. The rhizosphere is defined as the volume of soil adjacent to and influenced by the plant roots [as reviewed by Philippot et al. (2013)], relating intrinsically to plant health and soil fertility. Root exudates stimulate the growth of bacterial and fungal populations in the vicinity of the roots, consequently changing the dynamics of the nutrients in this region of the soil (Smalla et al. 2001; Aranda et al. 2011; Philippot et al. 2013). In this selective niche, bacterial communities are involved in processes that affect the plant life cycle, such as the fixation of atmospheric nitrogen (N_2), the production of phytohormones and the biocontrol of phytopathogens (Hardoim et al. 2011). Several studies have indicated that the structural and functional diversity of rhizosphere communities is affected by plant species due to differences in root exudation and rhizodeposition (Bertrand and Nalin 2001; Mendes et al. 2011; Dias et al. 2013; Philippot et al. 2013). Furthermore, effects of cropping practices (such as tillage, application of herbicides, bio-residue management), soil type, plant growth stage, genotype and other environmental factors also influence the composition of the microbial community in the rhizosphere (Smalla et al. 2001; Wu et al. 2008a; van Overbeek and van Elsas 2008; Andreote et al. 2010; Hardoim et al. 2011).

In vineyards, many management practices could influence soil characteristics, consequently modulating the grapevine root systems, and further impacting the overall assemblies of the microbial communities in this niche. Organic and regenerative management of soils has been widely regarded as conferring a positive effect upon soil biology, leading to a significantly higher level of biological activity, mainly promoted by reduced tillage, application of nutrients, and elimination of pesticide and herbicide inputs (Girvan and Bullimore 2003; Morlat and Jacquet 2003; Caravaca et al. 2005; Martins et al. 2012). The effects of agricultural land management on bacterial communities in the rhizosphere have been determined in several studies, mainly by cultivation-based techniques. Organic amendments were shown to significantly increase populations of soil bacteria antagonistic to tomato pathogens (Wu et al. 2008a). The source of plant nutrients (synthetically derived minerals vs. decomposed plant material) had a large effect on microbial communities (Bossio et al. 1998; Wu et al. 2008b).

However, the major problem of cultivation-based analysis is that only a small proportion of the bacterial community can be recovered from the rhizosphere (Lejon et al. 2008; Wu et al. 2008b). Such limitations can be overcome by analyzing DNA extracted from rhizosphere samples. Recently, the analysis of 16S rRNA gene fragments that were PCR amplified from community DNA was used to unravel bacterial communities in the rhizospheres of several plants (Wu et al. 2008a; Aranda et al. 2011; Hardoim et al. 2011; Dias et al. 2013). In the vineyard specifically, a cultivation-independent approach (automated ribosomal intergenic spacer analysis—A-RISA) was used

to demonstrate that bacterial communities in the soil were altered with the addition of organic compost (Saison et al. 2006). Dell'Amico et al. (2008) investigated vineyard soil by DGGE, and identified copper-tolerant bacteria, which are indicators of copper pollution in the soil (primarily from fungicides). Lejon et al. (2008) analyzed bacterial and fungal communities in soil from Japanese vineyards by cultivation-independent approaches, and revealed unique and extremely diverse communities in these soils. However, data describing the bacterial community structure and diversity in the rhizosphere of vineyards are lacking.

The objective of the present study was to examine the structure and the diversity of bacterial communities in the rhizosphere of vines (*Vitis vinifera* L.) cultivated in San Juan, comparing the assemblies of bacteria in different management systems (organic vs. conventional) by cultivation-independent techniques. The picture is composed by the fingerprinting comparisons, together with the quantification of total and N_2-fixing bacteria, and a description of the bacterial assemblies made by high-throughput sequencing of the 16S rRNA gene.

Materials and methods

Vineyard locations

Viticulture is widespread in the study region, with a prevalent cultivation of the variety *Syrah*, which was selected for this study. The soils used for the vineyards are normally pristine (without previous use for agriculture), stony and alluvial, with alternating levels of gravel, sand, silt, and clay. The climate of the region is arid, with an average temperature during the year of approximately 17 °C. Rainfall is scarce (90 mm per year), mainly concentrated from December (early summer) to May (autumn) (De Fina 1992; Bisigato et al. 2009), forcing the use of irrigation with either surface (rivers) or groundwater.

The vineyards located in the Valley of Tulum (center of San Juan, Argentina) were selected according to the farming system (organic and conventional), separated by a distance of 4 km. The vineyard under organic management is located in "Las Moras" (31°33′20.4″S and 68°24′0.4″W, 588 m altitude), and the vineyard managed using conventional farming methods is located in "Las Trojas" (31°39′43″S and 68°21′32.7″W, 577 m altitude). They shared very similar characteristics: soil type (sandy-loam), grape variety (*Syrah*), age (15 years), pruning system, canopy management, and sun exposure.

The conventional vineyards used a conventional tillage system, were fertilized with calcium, magnesium and zinc, and treated with several agricultural chemicals: glyphosate acid (round-up), and the fungicides Caurifix® WG (copper oxychloride) and Kumulus® DF (sulfur). The organic vineyards used annual applications of crushed pruned vine-wood (2 t/ha fresh weight).

Experimental procedure

In the year of 2012, the rhizosphere samples were collected under four distinct plants in each vineyard (total of eight plants sampled), at a layer between 40 and 50 cm depth, which is the soil layer harboring the majority of the root system in vineyards (Linsenmeier et al. 2010). This layer was also selected to avoid the physical effects of tillage on the composition of bacterial communities, and to determine the accumulated effects of differential management on the rhizosphere assembly. The samples were collected under aseptically conditions (sterile gloves, newly-sterile materials), being located very near the root growth, by sampling soil cores (with root pieces) using 70 % ethanol rinsed stainless steel shovels. The samples were immediately stored at −80 °C in plastic bags (in the dark) for subsequent DNA-based analyses.

The remaining rhizosphere soil was air-dried and sieved (2 to 5-mm mesh size) prior to physical and chemical analyses of the soil. These analyses were carried out in the Laboratory of Soil Analysis at Escola Superior de Agricultura Luiz de Queiroz (ESALQ/USP, Piracicaba, Brazil), according to the methodology described by Van Raij et al. (2001). The quantified properties of the rhizosphere samples were texture (sand:silt:clay), pH, and potassium (K), phosphorus (P), calcium (Ca), and magnesium (Mg) content (Table 1).

DNA extraction

The total DNA was obtained by extraction using the commercial kit Power Soil DNA Isolation (MoBio, Carlsbad, USA) according to the manufacturer's

Table 1 Physical and chemical parameters of rhizosphere soils from organic and conventional vineyards

Rhizosphere soil characteristics	Vineyards	
	Organic	Conventional
pH	6.93 ± 0.94A	6.60 ± 0.69A
P (mg dm^{-3})	33.67 ± 10.79A	19.50 ± 8.38B
K (mmolc dm^{-3})	5.52 ± 1.17A	5.22 ± 1.88A
Mg (mmolc dm^{-3})	17.67 ± 2.34A	15 ± 3.58A
Ca (mmolc dm^{-3})	305 ± 5.93A	268 ± 36.02 B
Sand:silt:clay (%)	64.2:22.4:13.4A	57.8:25.5:16.7A

The data are the means of four replications, followed by the standard deviation. Different letters in the lines indicates significant differences between values, as determined by Tukey's test ($p < 0.05$)

mmolc milimols of soils charges occupied by the element

instructions, yielding approximately 5.0 μg of DNA per sample. The DNA was evaluated by electrophoresis on a 1.0 % agarose gel in TAE buffer (400 mM Tris, 20 mM acetic acid, 1 mM EDTA). The gel was stained in a solution of ethidium bromide and visualized under UV light.

Quantification of targeted communities by quantitative PCR (qPCR)

The number of copies of the genes that encode for 16S rRNA and *nif*H (ubiquitous in nitrogen fixing bacteria) per gram of soil were determined by qPCR. The amplification of the 16S rRNA fragment was carried out with primer F (5′-CCT ACG GGA GGC AGC AG-3′) and primer R (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al. 1993) in PCR reactions of 25 μL containing 12.5 μl of Platinum® Quantitative PCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA), 0.4 mM of each primer and 1.0 μl (approximately 50 ng) of environmental DNA. Amplifications was performed with an initial denaturation at 95 °C for 3 min, followed by 35 amplification cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. PCR was carried out on a Rotor Gene 6,000 (Corbett Life Science, Australia).

The abundance of *nif*H genes was determined using the primers FGPH19 (Simonet et al. 1991) and PolR (Poly et al. 2001). The PCR reaction mix (25 μL) contained 12.5 μl of Platinum® Quantitative PCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA), 0.4 mM of each primer and 1.0 μl (approximately 50 ng) of environmental DNA. Amplifications were performed with one initial denaturation step at 95 °C for 5 min, followed by 35 amplification cycles of 94 °C for 1 min, 57 °C for 45 s, and an extension of 1 min at 72 °C. PCR was carried out on a Rotor Gene 6,000 (Corbett Life Science, Australia).

Standard curves were constructed by amplifying known quantities (10^2–10^7) of the target genes, which also served to determine the efficiency and regression (R^2) values for the detection systems. Melting curves were constructed for both genes, where amplification products were submitted to a range of temperature from 72 to 96 °C. Specificity of amplicons were determined by the use of specific primers and also the generation of bands with expected size, as observed in common electrophoresis. To test significant differences in the absolute quantification values (copies of the targeted gene per gram of soil), we performed a variance analysis (ANOVA) and further compared the average values using Tukey's test at a significance level of 0.05.

Analysis of the bacterial communities in rhizosphere soils by denaturing gradient gel electrophoresis (DGGE)

The analyses of the structures of the bacterial communities were performed by an amplification of the fragment V6 of the 16S rRNA gene utilizing the primers U968⊥GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA-3′) and 1378R (5′-CGG TGT GTA CAA CGC CCG GGA ACG-3′) (Heuer et al. 1997). Reactions were in a final volume of 25 μL, containing 1 μL of DNA template (approximately 50 ng), 1× Taq buffer, 2.50 mM $MgC1_2$, 0.25 mM of each dNTP, 0.4 mM of each primer, 1 % formamide, and 1 U AmpliTaq DNA polymerase

(Fermentas, São Paulo, Brazil). The amplifications were carried out in a Gene Pro thermal cycler (Bio-ER, China), with an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min.

To analyze specific bacterial groups, the initial PCR reactions were designed to target the 16S rRNA gene of *Alphaproteobacteria* and *Betaproteobacteria*, as described by Gomes et al. (2001), and also *Actinobacteria*, as described by Heuer et al. (1997). Each 25 µL reaction mixture for the first PCR amplification contained 1 µL of DNA template, and the PCRs were performed according to their respective protocols (Heuer et al. 1997; Gomes et al. 2001). The PCR products from this initial reaction were then used as the templates for the nested PCRs with primers U968⊥GC and 1378R, as described above.

For amplification of *nif*H, here used as a proxy for nitrogen fixing bacteria, the protocol was similar to that of Simonet et al. (1991) and Poly et al. (2001). A first PCR was carried out with the primers FGPH19 and Pol R. Each 25 µL PCR mixture contained 1 µL of DNA template (approximately 50 ng), 1× Taq buffer, 2.50 mM $MgC1_2$, 20X BSA, 2.5 mM of each dNTP, 100.0 pmol/µl of each primer and 5 U AmpliTaq DNA polymerase (Fermentas, São Paulo, Brazil). PCR assays were performed in a Gene Pro thermal cycler (Bio-ER, China). The cycling program consisted of an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR amplification products were used as the template for the second PCR with the primers PolF-GC and AQER94. Each 25 µl PCR mixture contained 1 µL of DNA template (approximately 50 ng), 1X Taq buffer, 2.50 mM $MgC1_2$, 2.5 mM of each dNTP, 100.0 pmol/µl of each primer and 5 U AmpliTaq DNA polymerase (Fermentas, São Paulo, Brazil). The cycling program consisted of an initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min.

The PCR results were always checked by agarose gel electrophoresis to determine amplicon size and specificity prior to DGGE analysis. DGGE analysis was performed in a PhorU-2 apparatus (Ingeny, Goes, the Netherlands) in 0.5 X TAE buffer (20 mM Tris–acetate, 1 mM EDTA, pH 8.0) and the gels were run at 200 V for 8 h at 60 °C. Gel casting was performed as described by Muyzer et al. (Muyzer et al. 1993), with a gradient consisting of 45–65 % denaturant (100 % denaturant contained 7 M urea and 40 % formamide) for analyses of total bacteria, *Alphaproteobacteria*, *Betaproteobacteria* and *Actinobacteria*. The gradient for *nif*H analysis was 40–65 %. After the run, the gels were stained with SYBR Gold nucleic acid staining solution (Invitrogen, Brazil) and the DGGE patterns were made visible by illumination with UV light. Images of the gels were obtained and analyzed using Image Quant TL 1D (Version 7.0) (GE Healthcare, General Electric Company).

Bands were detected and normalized, and the position and intensity of individual bands in the profiles (species parameter) were recorded. To assess the complexity of the bacterial communities, bands with similar motility (1 % tolerance) were assigned to the same band migration position. Then, matrices containing the relative area of each band in each sample were generated and used for further analyses.

Sample clustering was analyzed by principal coordinate analysis (PCoA), where the beta-diversity was used to compare samples using the matrices of band intensities as the input data. PCoA was determined using the software PAST (Hammer 2011), and the validation of samples clustering (valid those with $p < 0.05$) was performed by ANOSIM at Primer software (Clarke and Gorley 2006), based on 9,999 permutations. Then, to correlate the band patterns with environmental variables, a detrended correlation analysis (DCA) was undertaken, revealing the linear distribution of the data for all targeted groups. Based on those results, the redundancy analysis (RDA) was applied, with a significance test performed by the nonparametric Monte Carlo permutation test (with 499 random permutations). The results of the Monte Carlo test supplied information about the marginal effects of the environmental variables, quantifying the amount of variance explained by each factor (Lambda 1). These analyses were carried out using Canoco 4.5 software (ter Braak and Smilauer 1998; Andreote et al. 2009a).

Analysis of the bacterial communities by sequencing the V6 region of the 16S rRNA gene

The target region was amplified in four samples (two from each cultivation system). The DNA extracted

from each sample was used for amplification with the primers 967F and 1046R (Sogin et al. 2006), amended with two adapters for sequencing on an *IonTorrent* Personal Genome Machine (Life Technologies, EUA). The primer 967F received an additional distinct mark (tags of identification composed of five base pairs) for each sample. The PCR reaction conditions (50 μL) were composed of 1× PCR buffer, 3.0 mM $MgCl_2$, 200 μM of dNTP, 0.2 μM of each primer, and 0.02 U/μL AmpliTaq DNA polymerase (Fermentas, São Paulo, Brazil). Then, the amplification products were purified using the Charge Switch *PCR Clean-UP* kit (Invitrogen, Brazil), and subsequently subjected to sequencing by the *IonTorrent* Personal Genome Machine (Life Technologies, EUA), available in the Laboratory of Environmental Microbiology, CNPMA–Embrapa Meio Ambiente (Jaguariúna, Brazil).

Sequence analyses were carried out using QIIME (*Quantitative Insights Into Microbial Ecology*) (Caporaso et al. 2010), where sequences were separated by samples according to their tags, adapters and primer sequences were removed, and low-quality sequences (quality < 20) were discarded. The resulting sequences were binned into operational taxonomic units (OTUs) on the basis of 97 % similarity using the method *uclust* (Edgar 2010). Representative sequences for each OTU were further subjected to taxonomic analyses made using the BLAST method against the Greengenes database (*Caporaso Reference Otu*) (Caporaso et al. 2010), generating an OTU table.

To avoid bias caused by the different number of sequences analyzed from each sample, the OTU table was rarified at 10,680 sequences from each sample. The rarified data were then used to generate classification plots and to estimate ecological indices, such as phylogenetic distance (PD), diversity (Shannon) and richness (Chao1), in addition to determining the coverage (Goods coverage) obtained by the sequence effort used. A network view was also summarized in QIIME and visualized using CytoScape 3.01, combining the distribution of samples and OTUs, allowing the visualization of sample similarities and the composition of OTUs by sequences from both areas, or exclusively made of sequences from one of the vineyards.

In addition, the Simper test was performed on PAST (Hammer 2011) using the Chord algorithm, based on taxonomic ranks of phyla and classes, to determine the major groups contributing to the dissimilarities observed between samples from organic and conventional vineyards.

Results

Physical and chemical characteristics of rhizosphere samples

Most of the physical and chemical characteristics of the sampled rhizospheres were similar in the conventional and organic vineyards. The pH values were 6.93 ± 0.94 and 6.60 ± 0.69 for organic and conventional management, respectively. The K, Mg, sand, silt and clay contents were also statistically similar (Table 1). However, the contents of Ca and P were significantly higher in the organic vineyards compared with the conventionally managed fields ($p < 0.05$) (Table 1).

Quantification of 16S rRNA and nifH genes by real time PCR

16S rRNA genes and *nif*H genes were detected and quantified accurately, with efficiency values of 88 and 86 %, respectively; logarithmic regression curve (R^2) values of 0.99 (for both analyses) and amplification specificity was determined, with single peaks observed during the melting curve analysis. Statistically, similar quantities of 16S rRNA genes were observed between the conventional and organic vineyards ($p > 0.05$), with log values between 9.74 ± 0.35 and 9.67 ± 0.44 per gram of rhizosphere soil, respectively. The *nif*H gene copies also did not differ significantly between conventional and organic samples, with log values between 4.89 ± 0.61 and 4.79 ± 0.22 copies per gram of rhizosphere, respectively.

Community structure analysis based on PCR-DGGE

Analyses of the band profiles (total bacteria, *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria* and *nif*H) revealed qualitative and quantitative differences in samples from each vineyard system. In general, gels were composed of complex patterns formed by a high number of bands in all targeted

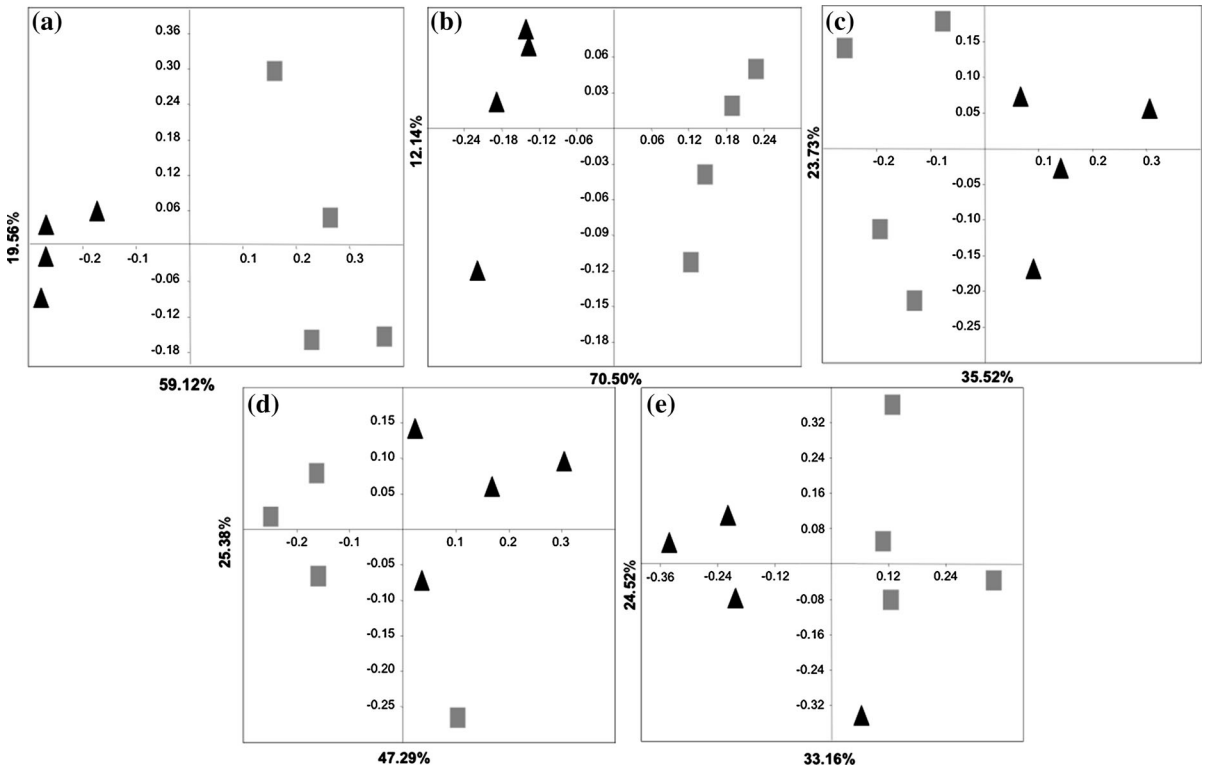

Fig. 1 Principal coordinate analyses (PCoA) based on DGGE patterns for the analysis of Bacteria (**a**), *Alphaproteobacteria* (**b**), *Betaproteobacteria* (**c**), *Actinobacteria* (**d**) and nitrogen-fixing bacteria (*nif*H-based) (**e**), present in organic and conventionally managed vineyards in Argentina. Values on *axes* indicate the percentage of variance explained in each axis

groups (Table S1). Although the visual separation of the samples from distinct vineyards is possible, more robust inferences were performed based on PCoA analyses (Fig. 1). PCoA plots revealed that all fractions from the bacterial communities share low levels of similarity in the rhizosphere of plants cultivated under distinct management systems. In all cases, the separation among samples from each vineyard is observed in the first axis (Fig. 1). These separations were based on high amounts of variance explained by the distribution of samples along the plots (from 57.7 to 82.7 % in analyses of *nif*H and *Alphaproteobacteria*, respectively) (Fig. 1). Moreover, the ANOSIM indicated that all separations were statistically significant ($p < 0.05$), clustering separately samples from organic or conventionally managed vineyards [R values varying between 0.70 (Betaproteobacteria) and 0.98 (Alphaproteobacteria)].

The correlation between the microbiological data and the physical and chemical characteristics of soils, determined by RDA, was evidence of the low variance due to changes in soil characteristics (Table 2). The correlation of calcium and magnesium in the structuring of *Betaproteobacteria* and *Actinobacteria* communities, together with the particular effect of calcium upon the community of bacteria harboring the *nif*H gene, was evidenced by RDA, and supported by the Monte Carlo permutation test ($p < 0.05$) (Table 2).

Sequence-based analyses of the bacterial groups in the rhizosphere of vineyards

A total of 67,830 valid sequences (average size of 56 bp) were generated; 43,542 and 24,288 from organic and conventional samples, respectively (summing the totals from the two repetitions per treatment). The taxonomic affiliations of the sequences revealed a high proportion of reads affiliated as unclassified Bacteria (28.8 and 18.0 % for organic and conventional vineyards, respectively). Other sequences were allocated into 30 bacterial phyla, with 13 represented by more than 0.1 % of sequences in at least one of the

samples sequenced. The most abundant phyla found in both vineyards were *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteriodetes*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia* and *Planctomycetes* (more than 1 % of sequences in at least one sample sequenced) (Fig. 2a). Comparing the differences in the abundance of these phyla, organic vineyards revealed a higher abundance of sequences affiliated with *Firmicutes*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes*, while samples from the conventionally managed vineyard had higher numbers of sequences related to *Proteobacteria* and *Bacteroidetes* (Fig. 2a).

Distinctions in the composition of bacterial communities were also observed at the class level. Clear distinctions were observed for classes within the phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria* (Fig. 2b–f). Among sequences affiliated with *Proteobacteria*, *Gammaproteobacteria* dominated in the conventional vineyard, and *Alphaproteobacteria* was present at a higher proportion in the organically managed vineyard (Fig. 2b). The relative abundance of classes in other phyla also differed between the conventional and organically managed vineyards, with the exception of the *Firmicutes*, in which *Bacilli* dominated in both types of vineyards (Fig. 2e).

Although differences in the taxonomic affiliation of sequences is visible in Fig. 2, Simper was used to determine which bacterial groups contributed most to the differences between the microbial communities in the different vineyards. Among the phyla contributing most to the differences in the bacterial assemblages (responsible for more than 1 % of the distinction) are the most abundant ones, except for *Planctomycetes* (Table 3). Together, the variation of the frequencies of these phyla explains 61.13 % of the difference between the grapevine fields. Shifts in minor phyla are responsible for 15.23 % of the variation, and shifts in values for unclassified bacteria account for 22.12 % of the variation (Table 3).

For the class-related differentiations within the phylum *Proteobacteria*, *Gammaproteobacteria* explained more than 10 % of the variation, followed by *Alphaproteobacteria* and *Betaproteobacteria* (7.82 and 7.80 %, respectively). More than 5 % of the variation was due to *Actinobacteria* (*Actinobacteria*) and *Bacilli* (*Firmicutes*), with values of 5.87 and 6.42, respectively (Table 4). Another 10 classes, in addition to unclassified *Proteobacteria*, unclassified *Actinobacteria*, unclassified *Firmicutes* and unclassified *Verrucomicrobia*, explained more than 0.5 % of the variation, accounting for 58.15 % (Table 3).

In addition to the analyses based on the affiliation of sequences to taxonomic ranks from databases, the sequences were also analyzed on the basis of clustering into OTUs. The rarified datasets were used to assign 42,720 sequences (10,680 per samples) into 12,218 OTUs. Based on the distribution of sequences into OTUs, high values for coverage were observed in all samples, ranging between 0.84 and 0.86 in samples collected in conventional vineyards, and between 0.78 and 0.82 in samples from the organically managed field. The resulting OTU table was also used to generate values of ecological estimators for samples

Table 2 Statistical significance of environmental variables determined by multivariate analysis based on the redundancy methodology (RDA)

	16S rRNA		Alphaproteobacteria		Betaproteobacteria		Actinobacteria		*nifH*	
	Lambda 1	p value	Lambda 1	p value	Lambda 1	p value	Lambda 1	p value	Lambda 1	p value
Calcium	0.184	0.192	0.184	0.258	***0.248***	***0.014***	***0.237***	***0.014***	***0.227***	***0.004***
Magnesium	0.199	0.164	0.181	0.268	***0.251***	***0.014***	***0.220***	***0.046***	0.181	0.240
Potassium	0.115	0.564	0.110	0.670	0.205	0.124	0.151	0.418	0.113	0.732
Phosphorus	0.218	.0130	0.181	0.258	0.121	0.646	0.176	0.248	0.162	0.350
pH	0.169	0.314	0.136	0.432	0.128	0.602	0.139	0.504	0.171	0.244
H + Al	0.152	0.364	0.160	0.334	0.12.	0.676	0.151	0.432	0.168	0.252
Clay	0.174	0.274	0.169	0.282	0.172	0.296	0.178	0.214	0.165	0.304
Sand	0.216	0.132	0.216	0.116	0.171	0.304	0.200	0.134	0.178	0.212
Silt	0.249	0.054	0.232	0.076	0.131	0.514	0.181	0.222	0.180	0.190

Values in *bold-italicized* are statistically significant factors correlated to the composition of bacterial communities in the rhizosphere according to the Monte Carlo permutation test. Significant variables ($p < 0.05$) are presented in *bold*

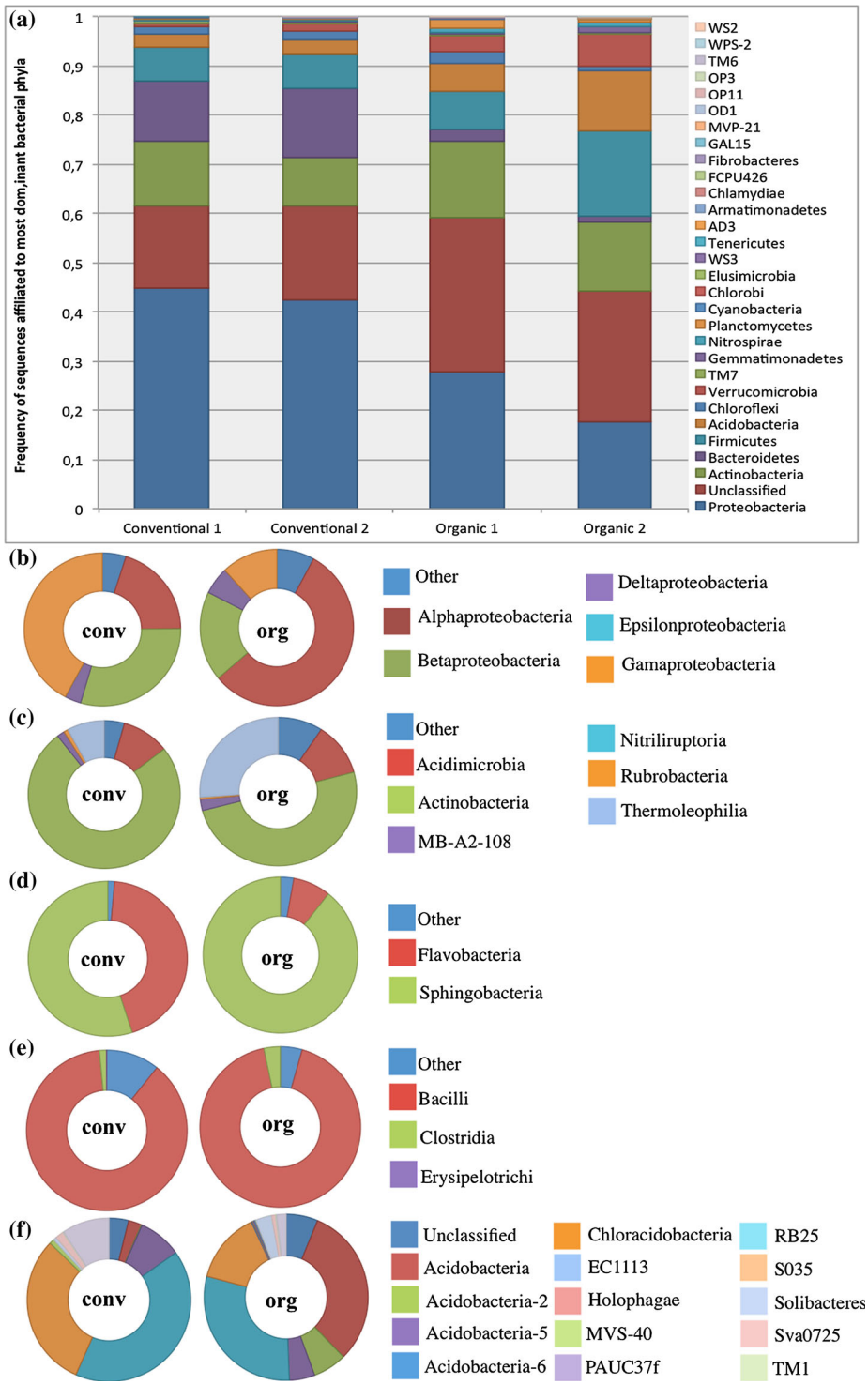

Fig. 2 Taxonomic affiliation of ribosomal sequences obtained from the rhizosphere of plants cultivated under organic or conventional management systems in vineyards. Values represent the percentage of affiliation into distinct phyla (**a**) or classes within the phyla *Proteobacteria* (**b**), *Actinobacteria* (**c**), *Bacteroidetes* (**d**), *Firmicutes* (**e**) and *Acidobacteria* (**f**)

from both vineyards (Table 4), suggesting a higher microbial diversity in the organically managed area. Diversity, as determined by the Shannon values, ranged between approximately 9.51 and 10.26 for the conventional and organic areas, respectively, together with the estimated values for richness, with average values of 5,426 and 7,260 for the same vineyards (Table 4). The phylogenetic diversity (PD) was also higher in the organic field in comparison to the conventionally managed area, as revealed by the phylogenetic distance.

The network plot of samples and OTUs revealed a clear separation among samples collected in each vineyard (Fig. 3), indicating that the differences observed in taxonomy-based analysis (Fig. 2; Table 4) are also present in the OTU-based approach. The vast majority of OTUs are composed of sequences from one or two samples, as indicated by the dense radiation to the borders of the network figure, while some OTUs were represented by sequences in all treatments (245 OTUs) (Fig. 3). These observations reinforce the previous analyses, confirming the clear distinction between samples from each vineyard.

Table 3 Affiliation of major taxonomic groups correlated with the differentiation of the bacterial community composition in vineyards cultivated under organic or conventional practices, according to the determination based on the Simper test

Phyla	%	Classes	%
Proteobacteria	28.88	Gammaproteobacteria	10.16
		Betaproteobacteria	7.82
		Alphaproteobacteria	7.80
		Deltaproteobacteria	1.15
		Unclassified	1.94
Actinobacteria	9.72	Actinobacteria	5.87
		Thermoleophilia	1.83
		Acidimicrobiia	0.98
		Unclassified	0.83
Firmicutes	7.22	Bacilli	6.42
		Unclassified	0.56
Bacteroidetes	6.99	Sphingobacteriia	3.85
		Flavobacteriia	3.03
Acidobacteria	4.53	Acidobacteria	1.47
		Acidobacteria-6	1.35
Verrucomicrobia	2.74	Spartobacteria	1.07
		Pedosphaerae	0.66
		Unclassified	0.81
Chloroflexi	1.05	Anaerolineae	0.55
Others	15.23		
Unclassified bacteria	22.12		

Only phyla and classes explaining more than 1.0 and 0.5 % of distinction, respectively, are listed

Discussion

Plant species (even specific genotypes or varieties), soil type and agricultural practices are the most important drivers of the composition of the microbial community in the rhizosphere (Wu et al. 2008b; Aranda et al. 2011; Dias et al. 2013). The analysis of microbial communities in the rhizosphere of plants cultivated under different management systems (organic vs. conventional) offers an important opportunity to explore the relationships between biotic and abiotic factors acting in this niche. Different crop management styles can result in a differential composition of substrates that will ultimately determine, by promotion or inhibition, the establishment of different microbial groups in the rhizosphere (Silva et al. 2013; Philippot et al. 2013). In this study, it was determined that alterations in the composition of bacterial

Table 4 Ecological estimators for the rhizosphere of conventional and organic managed vineyards

Vineyards	OTUs	Richness (Chao1)	Diversity (Shannon)	Phylogentic distance	Coverage (Goods)
Conventional 1	2,794	5,881	9.44	63.3	0.84
Conventional 2	2,768	4,970	9.57	54.2	0.86
Organic 1	3,240	6,692	10.11	62.5	0.82
Organic 2	3,664	8,549	10.40	71.1	0.78
Conventional (avg)	2,781	5,426	9.51	58.75	0.85
Organic (avg)	3,452	7.620	10.26	66.8	0.80

Values were determined based on rarified datasets with 10,680 sequences each

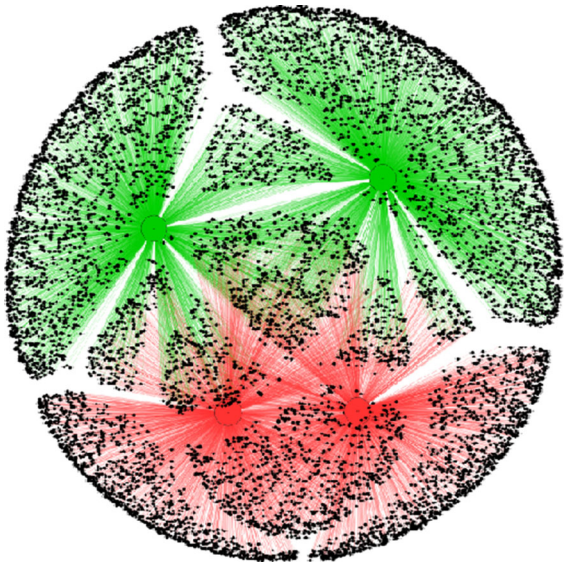

Fig. 3 Distribution of sequences retrieved from organic or conventionally managed vineyards, into OTUs (*black spots*) determined at 97 % of similarity. In addition, the OTUs are plotted indicating the common occurrence of sequences from both treatments, or the exclusive composition of OTUs by sequences from one vineyard or one repetition. The samples are plotted with the nearest locations indicating higher similarity values between each other

assemblages in the rhizosphere of vineyards is related with the use of different agricultural practices. The exploration of the arid environment where vineyards are cultivated is innovative, and the link with an important crop for the Argentinean economy makes this assessment very important for understanding the microbial ecology of the rhizosphere in the target system.

The physical and chemical characterization of soils demonstrated only small differences in parameters between the vineyards. Although not all possible variables were determined, these aspects of the soils are not greatly different. It was expected that soils under organic management would contain higher concentrations of nutrients, or lower pH values (Bronick and Lal 2005). However, the particularities of the studied area (location in a very dry area) and the proximity of areas where vineyards are cultivated, might explain the small differences observed.

Another similarity was observed in the quantification data, indicating that the targeted genes, bacterial 16S rRNA and *nif*H, are at similar amounts in the rhizosphere of samples under distinct agricultural management. The values are in accordance with the literature (Prosser and Nicol 2008; Silva et al. 2013), and the low variation in the amount of bacteria was expected. However, we expected to find shifts in the values of the *nif*H gene because soils harboring higher amounts of organic compounds are likely to harbor more abundant communities of nitrogen-fixing bacteria (Mäder et al. 2002; Silva et al. 2013). In this scenario, nitrogen fixation would play a pivotal role in nitrogen uptake in combination with the carbon present in organic matter, facilitating its degradation (Bronick and Lal 2005; Dias et al. 2012). Indeed, in the present study, the organic management is based on the addition of crushed pruned vine-wood to the soil, a residue with a high content of carbon and small amounts of nitrogen. It is possible that the layer of soil used for sampling could have masked its effect, with the selection for diazotrophs limited to the superficial layers of the soil.

The structure of the bacterial communities was very responsive to the adoption of distinct agricultural practices, with a clear differentiation based on DGGE and sequence-based analyses. All five systems (four based on taxonomic groups and one based on specific function-related groups) used to determine the structure of bacterial communities by DGGE revealed clear differences in the patterns of the rhizosphere of plants from each vineyard. This is a convincing indication that the selection exerted by agricultural practice is strong, and it might overcome the selection exerted by the plant upon the soil bacterial community, to compose the microbiome living in the rhizosphere. However, to better understand how the differences in this niche can lead to differential assemblages of bacteria in the rhizosphere, a distinction between the plant role and the distinctions attributed to soil properties is needed. One possible method is based on multivariate analysis, used in this study to correlate the microbiological data with rhizosphere soil parameters. The RDA indicates that specific changes in bacterial communities could be partially explained by soil characteristics. Differences in cations, such as calcium and magnesium, seem to play important roles in communities of *Betaproteobacteria* and *Actinobacteria*, possibly because of the lower selection of these groups by plant activities, such as observed for *Alphaproteobacteria*, a responsive group for shifts in plant metabolism (Andreote et al. 2009b).

Additionally, in arid soils, Ca^{++} and Mg^{++} carbonates precipitate to form secondary carbonate coatings, changing the particle organization, and interfering with soil structure (Zhang and Norton 2002). This might lead to the selection of bacterial communities by, for example, changing the soil atmosphere. In the case of *nif*H-based analysis, particularly affected by magnesium content, the most plausible explanation is that shifts in magnesium content and form result from a differential degradation of organic matter (qualitative and quantitative) (Boix-Fayos et al. 2001; Bronick and Lal 2005), which might result, among other factors, from a differential functioning of nitrogen-fixing bacteria.

The results of sequence-based analysis confirmed the trends observed in the DGGE approach, and identified the major bacterial groups living in the rhizosphere of the assessed plants, complementing the data from previous studies on the bacterial community structure of vine soils (Saison et al. 2006; Dell'Amico et al. 2008; Steenwerth et al. 2008). By using high-throughput sequencing, we detected more taxonomic groups than described in previous studies. A similar approach, based on soils under vine cultivation, identified the major groups as *Proteobacteria* and *Acidobacteria*, followed by *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Planctomycetes*, *Nitrospira*, *Cyanobacteria*, *Firmicutes* and *Verrucomicrobia* (Lejon et al. 2008). Comparing the rhizosphere structure with those results, it is possible to see similarities, and to determine the responsive groups for the presence of plant roots, clearly indicated by the phyla *Firmicutes*, detected in lower quantities in the study of Lejon et al. (Lejon et al. 2008) but the most abundant in the present survey of the rhizosphere. More deeply, comparing the rhizosphere profiles of the organic and conventional vineyards, we found clear distinctions in the bacterial assemblages. Based on taxonomic analysis, the Simper test identified the bacterial phyla and classes that are major contributors to the differences between samples, attributing the distinction between samples to shifts in the most frequent groups.

The complementation of the taxonomic-based analyses using OTU-based approaches confirmed the differences between the rhizosphere of each vineyard and lends to the discussion of differences in ecological estimators. A higher diversity of bacteria is present in the rhizosphere of organically managed grapevines, supported by richness, diversity and phylogenetic diversity. These results agree with previous studies, which found significantly higher microbial diversity in soils under organic management than in those managed using conventional systems (Mäder et al. 2002; Silva et al. 2013).

This observation is likely to be related to two major issues: the input of organic matter, which also acts as a source for inoculation of exogenous microbial cells in the organically managed area, and the susceptibility of agricultural systems to the establishment of exogenous (invader) organisms. The first explanation relies on the high occurrence of microbial cells in residues commonly used in organic agriculture (Saison et al. 2006; Puglisi et al. 2013). These microbes are resident in the organic matter during its generation (formation of animal and plant tissues), or can be acquired during the processing of crude organic matter; for example, during composting processing, performed prior to the addition to soils (Saison et al. 2006). The second issue is correlated with the ability that exogenous organisms have to survive in the soil, which is linked to the natural soil biodiversity (van Elsas et al. 2012). If we consider the values for ecological estimators, it is possible to suggest that the combination of both issues can result in fractions of microbial communities found in the organically managed vineyard originate from the input of organic compounds. Additionally, because their origin is linked with the generation and processing of organic matter, it is likely that it might replace the fractions of the bacterial communities lost along the continuous cultivation of grapevines (monoculture). In this sense, it certainly increases the repertoire of microbes that might compose the rhizosphere of plants, resulting in the differential composition of the rhizosphere bacterial communities in each system.

In summary, this study reported a large bacterial diversity and a responsive bacterial community in the rhizosphere of organically and conventionally managed vineyards. The results indicate that the plant selection exerted upon a differential set of bacterial cells is a major driver, upon soil characteristics and the bacterial assemblages in the rhizosphere of vineyards. Several questions remain to be answered, primarily linking these observations with important features attributed to bacterial-associated communities, such as the role of these differential assemblages on plant development and soil suppressiveness.

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