Plant Soil (2018) 424:405–417
https://doi.org/10.1007/s11104-017-3548-7

REGULAR ARTICLE

Microorganisms reveal what plants do not: wheat growth and rhizosphere microbial communities after *Azospirillum brasilense* inoculation and nitrogen fertilization under field conditions

Luciana P. Di Salvo · Lucía Ferrando · Ana Fernández-Scavino · Inés E. García de Salamone

Received: 4 July 2017 / Accepted: 21 December 2017 / Published online: 2 January 2018

Abstract
Aims *Azospirillum brasilense* is one of plant growth promoting bacteria used to improve plant growth and grain yield of cereal crops. The level of inoculation response is defined by complex plant-microorganism interactions, many of them still unknown. Thus, we evaluated both agronomic response and microbial ecology of wheat crop under *A. brasilense* inoculation and nitrogen fertilization at field conditions in order to improve inoculation efficiency.
Methods Treatments were: control, nitrogen fertilization and inoculation with 40M and 42M strains. Functional and structural diversity of rhizosphere bacterial communities were evaluated by community-level physiological and terminal restriction fragment length polymorphism profiles. Besides, aerial biomass, grain yield and counts of microaerophilic diazotrophic rhizobacteria were determined.
Results Plant ontogeny modified the number of culturable microaerophilic diazotrophic rhizobacteria. Although agronomic response did not show differences, plant ontogeny and the agricultural practices modified both physiology and genetic structure of rhizosphere microbial communities. Interestingly, these differences due to the treatments were observed at jointing stage but not at grain-filling stage of wheat.
Conclusions Our results demonstrate how different management decisions can change plant- microorganism relationships. While wheat could not show differences between some agricultural treatments, under the soil surface microbial communities could show them.

Keywords *Triticum aestivum* · CLPP · Crop production, Functional diversity · Structural diversity · T-RFLP

Responsible Editor: Hans Lambers.

L. P. Di Salvo
CONICET - Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires. Av. San Martín 4453, 1417 Ciudad Autónoma de Buenos Aires, Argentina

L. Ferrando · A. Fernández-Scavino
Cátedra de Microbiología, Facultad de Química, Universidad de la República, Gral. Flores, 2124 Montevideo, Uruguay

I. E. García de Salamone (✉)
Facultad de Agronomía, Departamento de Biología Aplicada y Alimentos, Universidad de Buenos Aires, Cátedra de Microbiología Agrícola. Av. San Martín 4453, 1417 Ciudad Autónoma de Buenos Aires, Argentina
e-mail: igarcia@agro.uba.ar

Introduction

Wheat, rice and maize provide more than 60% of calories of human diet, by direct consumption or livestock feed and subproducts production (FAOSTAT 2012). As food demand increases, crop production should intensify and more inputs, such as chemical fertilization, will be necessary as it was proposed in the paradigm of the green revolution occurred during the second half of the twentieth century. It is now known that this productive system could produce negative effects on the environment (Tilman et al. 2002), despite some agriculture

practices, such as no-tillage and crop rotations with cereal plants, which ensure a positive balance of soil nutrients and lower soil organic matter degradation (Grandy et al. 2006; Sisti et al. 2004). For this reason, nowadays, many authors propose a "new green revolution" in order to increase grain yields through both better understanding of plant-soil interactions and management of beneficial soil microorganisms (Den Herder et al. 2010; Gewin 2010).

Within these beneficial soil microorganisms, plant growth promoting rhizobacteria (PGPR) produces beneficial direct and indirect effects on plant growth (Bashan and de-Bashan 2010). *Azospirillum* is one of most studied PGPR genera. It has been well demonstrated that *Azospirillum* spp. inoculation increases growth, development and grain yield of several crops (Cassán et al. 2015). In addition, *Azospirillum* spp. increases fertilizer use efficiency under different soil and climatic conditions (Casanovas et al. 2015).

The level of inoculation response has been described as a wide range, determined by inoculant-plant-environment interactions (Cummings 2009; García de Salamone 2012a) which also determine microbial diversity in the rhizosphere (Philippot et al. 2013). During the last years, many authors have been looking for the relationships between aboveground and belowground diversity (Hooper et al. 2000; Mendes et al. 2015; Saleem and Moe 2014) in order to preserve species, and ecosystem processes and services (Hooper et al. 2000), including crop production (Philippot et al. 2013). For this reason, in this work both environments were considered in order to analyze inoculation and fertilization wheat response.

Due to inoculant-plant-environment interactions produce inconsistencies of the responses to *A. brasilense* inoculation, as it has been well mentioned before (Cassán and Díaz-Zorita 2016), the aim of this work was evaluated both agronomic response and microbial ecology of wheat crop under *A. brasilense* inoculation and nitrogen fertilization at field conditions in order to improve inoculation efficiency. The hypothesis of the work was that *A. brasilense* inoculation and N fertilization increase biomass production and grain yield of wheat and do not modify rhizosphere microbial communities under field conditions. Thus, agronomical response of wheat crop to *A. brasilense* inoculation and N fertilization at field conditions was evaluated. In addition, functional and structural diversity of rhizosphere communities were evaluated by analyses of culturable diazotrophic communities, community-level physiological profiles (CLPP) and terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA gene. Many authors emphasize the usefulness of combining various techniques, including cultivable traditional techniques, for the evaluation of soil microbial diversity (Bastida et al. 2008; Gil-Sotres et al. 2005; Kirk et al. 2004; Torsvik and Øvreås 2002). In this regard, some authors combined some techniques, such as physiological and molecular profiles, in order to analyze differences between plant species (Houlden et al. 2008; Miethling et al. 2000), soil type (Bossio et al. 2005; Miethling et al. 2000) and agricultural practices (Bossio et al. 2005; García de Salamone et al. 2012; Naiman et al. 2009; Xue et al. 2008). For this reason, both physiological and molecular approaches were used in this work in order to characterize the microbial diversity of wheat rhizosphere.

Materials and methods

Field site and climate conditions

An experiment was performed at field conditions (35°02′36" S and 59°37′59" W) in Villa Moll, Navarro, province of Buenos Aires, Argentina. This region experiences warm and humid weather with an average temperature 16.4 °C and average annual rainfall of 1037 mm. The wheat crop was conducted under rainfed conditions. The average rainfall during the crop season is 427 mm, and the rainfall during the field trial was 473 mm. The soil was a Chacabuco sandy loam (Aquic Argiudoll) and chemical characteristics of the upper soil layer (20 cm) before sowing were: pH (6.1 of 1:2.5 soil:water), electrical conductivity (0.45 dS m^{-1}), total organic matter (3.9%), nitrate (59.7 mg kg^{-1}), and available phosphorus (5.37 mg kg^{-1}).

Sowing and crop management

The wheat cultivar used was Klein Castor (Klein™, Alberti, Buenos Aires, Argentina). Before sowing, weed control was done with a unique combined application of 1.7 L ha^{-1} of glyphosate (Touchdown™ Syngenta™, Pilar, Buenos Aires, Argentina) and 5 g ha^{-1} of metsulfuron methyl (Metsulfurón 60 Zamba™, Villa Moquehuá, Buenos Aires, Argentina). The crop sequence of the plot was soybean, wheat/soybean, and

maize under no-tillage system for more than ten years. Sowing occurred on 18 July 2009, based on environmental conditions typical for the location. Seeds were sown at 150 kg ha^{-1} with row spacing of 17.5 cm. At sowing, the entire experimental plot was fertilized with 20 kg ha^{-1} of P as triple superphosphate. Crop management conditions were the same as those applied in adjacent fields. At tillering stage, the following herbicides were applied: 200 ml ha^{-1} of dicamba (Dicamba Zamba™, Villa Moquehuá, Buenos Aires, Argentina) and 5 g ha^{-1} of metsulfuron methyl (Metsulfurón 60 Zamba™, Villa Moquehuá, Buenos Aires, Argentina).

Experiment design and treatments

The experiment had a completely randomized block design with three treatments: Control, without N fertilization or inoculation, Fertilized (with 46 kg N ha^{-1} of Solmix™ − 28% N and 2.6% S- PASA Fertilizantes, Petrobras™, Bahía Blanca, Buenos Aires, Argentina), and Inoculated (with a combination of two different *A. brasilense* strains). Four blocks were applied perpendicularly to the topographic slope which was less than 0.5%. Twelve plots were considered in the experiment and the dimensions of each plot were 12.5 m by 3.25 m. Nitrogen fertilization was performed at beginning of the tillering stage. Inoculation treatment was carried out on the day of sowing, applying the inoculant to a rate of 15 ml kg^{-1} of seeds. The inoculant used in this experiment was a liquid formulation of NFb medium with 3.7×10^8 CFU ml^{-1} of two different *A. brasilense* strains. These strains were 40M and 42M (GenBank accession number HM002661 and HM002662, respectively). These strains were cultured separately and mixed using a 1:1 ratio (*v*/v) of each strain culture 24 h before using to inoculate the wheat seeds. Both strains were previously isolated from maize rhizosphere (García de Salamone and Döbereiner 1996), identified (García de Salamone et al. 2010) and vastly characterized (Di Salvo et al. 2014). These strains have shown different fatty acid methyl esters composition and indolic compounds production (Di Salvo et al. 2014). Previously, it has been demonstrated major inoculation response with multi-strain inoculants than monoaxenic inoculants in wheat (García de Salamone 2012b; Hungria et al. 2010), maize (García de Salamone and Döbereiner 1996) and rice (García de Salamone et al. 2010).

Sampling and determinations

Rhizosphere soil, roots and aerial parts of the wheat plants were sampled at jointing (88 days after sowing, DAS) and grain-filling (133 DAS) stages of the crop. At physiological maturity (154 DAS) samples of aerial biomass were taken. At the three phenological stages, aerial parts were sampled by cutting all the plants which were growing in a line of 0.5 m (García de Salamone et al. 2012). Each composite sample had an average of seventeen plants per each plot. Every line was randomly selected in each plot as representative of the canopy, avoiding the border effects. In order to determine aerial biomass, sampled wheat plants, excluding their reproductive structures, were dried to constant weight at 55 °C. Samples of rhizosphere soil and roots were taken with a core on the seeding line at a depth of 0–20 cm. A total of twelve samples of both aerial biomass and rhizosphere soil with roots were taken at each ontogenetic stage of the wheat crop.

Microbial counts and analysis of functional diversity

Roots were manually separated from rhizosphere soil of the samples obtained at jointing and grain-filling stages. Roots were used to perform soil suspensions in aqueous solution of NaCl (9 g L^{-1}). Ten-fold dilutions were prepared for each sample. CLPP were analyzed using 96-well microplate with 23 different sole-carbon sources, as described previously by Di Salvo and García de Salamone (2012). Microplates were inoculated with 50 μl from the 10^{-4} dilution and were incubated at 30 °C for 96 h. Absorbance values were taken every 24 h with a microplate reader Multiskan EX™ (Labsystems, Vantaa, Finland) at 590 nm. Absorbance values from 48 h of incubation were used to perform further analyzes described below. Ten-fold dilutions were also used to analyze the most probable number (MPN) (de Man 1983) of diazotrophic rhizobacteria, using N-free NFb semisolid medium (Döbereiner and Pedrosa 1987).

Analysis of structural diversity

Rhizosphere soil samples were dried at 55 °C to preserve samples (García de Salamone et al. 2012). Total DNA was extracted from 0.5 g of each sample with the UltraClean Soil DNA Isolation Kit (#12800, Mo Bio™ Laboratories, Carlsbad, CA, USA). Extracted DNA from rhizosphere soil samples were used as templates

for 16S rRNA gene amplification with 27f (5'-AGAGTTTGATCMTGGCTCAG-3′) and 1392r (5'-ACGGGCGGTGTGTRC-3′) primer pairs (Marchesi et al. 1998). The 27f primer was 5′ labeled with 6-carboxyfluorescein. Three aliquots per sample of the extracted DNA were used to perform PCR amplification. All PCR were carried out in 25 μL (total volume) solutions containing 100 ng of DNA solution, 0.1 mM of each primer, 2.5 mM of $MgCl_2$, BSA (0.2 mg mL^{-1}), Taq 1× buffer, 0.2 mmol L^{-1} of each dNTP, and 0.4 U mL^{-1} of Taq DNA polymerase (Invitrogen™, Carlsbad, CA, USA). The reactions were performed in a thermocycler (GeneAmpPCR System 2400™, PerkinElmer™, Waltham, MA,USA) using the following steps: initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 1 min each one, 55 °C for 1 min, and 72 °C for 3 min, with a final extension step at 72 °C for 10 min.

The three aliquots of each sample were pooled after PCR amplification and the labeled PCR products were concentrated and desalted with centrifugal filter columns (Microcon 100™, Amicon™, EMD Millipore™, Billerica, MA, USA). Approximately 300 ng of labeled PCR products were digested with *Msp*I and *Hha*I (Fermentas™, Thermo Fisher Scientific™, Waltham, MA, USA). After enzyme inactivation by heating at 65 °C for 15 min, samples were sent to Macrogen Korea for GeneScan Service using the GS1200LIZ DNA fragment length standard (Applied Biosystems™) to assess the length of fluorescently labelled terminal restriction fragments (T-RFs).

The different T-RFs sizes obtained by capillary electrophoresis were analyzed by PeakScaner v1.0 software™ (Applied Biosystems™). All the T-RFs sizes between 50 and 550 bp, with peak heights larger than 100 fluorescence units, were considered for the analysis to obtain reproducible T-RFLP profiles. As enzymatic restrictions were performed with two different enzymes, two data sets of electropherograms were obtained. Total fluorescence intensity present in each electropherogram was compared within each data set; T-RFLP data was standardized to the lowest quantity, as described by Dunbar et al. (2001). All profiles were aligned. Results were contrasted with *in silico* digestion made using the virtual digest (ISPaR) of the Microbial Community Analysis III (MiCA) Software -online available- with primer sequences and restriction enzymes used in this work and the database named RDP (R10, U27) 700,829 Good Quality (>1200) Bacterial (Shyu et al. 2007). Sequences of "unculturable bacteria" were discarded for these analyses.

Statistical analyses

Functional diversity was analyzed using the Shannon's Index (*H* index) which was obtained from absorbance values of CLPP microplates. The *H* index was calculated as $H = -\sum p_i\ (\ln p_i)$ where p_i is the ratio of the activity on each substrate to the sum of activities on all substrates (Gómez et al. 2004). In all cases, activity was the absorbance value on each substrate from 48 h of incubation. In order to evaluate structural diversity, the *H* index was calculated using T-RFLP data. In this case, the *H* index was calculated as $H = -\sum p_i\ (\ln p_i)$ where p_i is the ratio of the height of peaks for each size of fragment to the sum of height of all peaks, adapted from Gómez et al. (2004). T-RFLP and CLPP profiles were analyzed using discriminant analysis analyses. Fluorescence intensity, standardized as described before, and absorbance values were used to perform the discriminant analyses of T-FRLP and CLPP, respectively. *H* index from both CLPP and T-RFLP data, aerial biomass and grain yield data were analyzed by ANOVA and Tukey's test at $P \leq 0.05$ for mean comparisons. All the analyses were performed with the software INFOSTAT/Professional 1.1 (Di Rienzo et al. 2011).

Results

Aerial biomass, grain yield and culturable diazotrophic rhizobacteria of wheat plants.

Table 1 summarized mean values of grain yield and aerial biomass at three phenological stages of wheat plants. No significant differences between treatments were observed at any phenological stage. Table 2 shows the MPN of diazotrophic bacteria in the rhizosphere of wheat plants. The ontogeny of wheat plants modified the MPN of diazotrophic rhizobacteria but inoculation and fertilization did not modify the number of this physiological group at any phenological stage (Table 2).

Characterization of functional diversity of the bacterial communities in the rhizosphere of wheat plants.

Figure 1 shows the discriminant analysis of the CLPP of bacterial rhizosphere communities at both phenological stages. Axis 1 and Axis 2 explained the 97% of the total variation. Microbial communities clustered on the right of Axis 1 used preferentially

Table 1 Aerial biomass and grain yield of wheat at three phenological stages of plants under different treatments

Phenological stage	Agronomic variable	Treatments		
		Control	Inoculated	Fertilized
		(kg ha^{-1}) ± standard deviation		
Jointing	Aerial biomass	4335 ± 1380 a	4442 ± 1218 a	4282 ± 537 a
Grain-filling	Aerial biomass	13,307 ± 3430 a	16,319 ± 2653 a	16,119 ± 1963 a
Physiological maturity	Aerial biomass	7142 ± 1355 a	7403 ± 1530 a	7492 ± 1307 a
	Grain yield	5514 ± 1166 a	5526 ± 1309 a	5914 ± 1023 a

Different letters indicate significant differences with Tukey's test ($P \leq 0.05$) between treatments at each phenological stage with $n = 12$

citric acid, mannitol and phenylalanine as carbon sources while microbial communities clustered on the left used preferentially glycine and dextrose. Microbial communities clustered on the top of Axis 2 used preferentially glutamine and xylose as carbon sources while microbial communities clustered on the bottom used preferentially citric acid and histidine. The discriminant analysis showed that the microbial communities clustered mainly by the phenological stage of the wheat plants and secondly by the inoculation and fertilization treatments applied on these plants (Fig. 1). According to this, treatments did not modify *H* index but phenological stages modified the functional diversity (Table 3).

Characterization of structural diversity of the bacterial communities in the rhizosphere of wheat plants.

Digestions of the 16S rRNA amplified products with the *Hha*I and *Msp*I restriction enzymes produced 13 and 26 different T-RFs sizes, respectively. Of these, 5 and 18 T-RFs sizes obtained with each enzyme respectively were observed in both the rhizosphere of control plants and in the rhizosphere of treated wheat plants. Some of these T-RFs sizes were the most abundant in the rhizosphere of wheat plants under the three different treatments (Fig. 2).

Figure 3 shows the discriminant analysis of the T-RFLP of bacterial rhizosphere communities at both phenological stages. Axis 1 and Axis 2 explained the 75% of the total variation. Microbial communities on the right of Axis 1 clustered mainly by the 144 bp, 148 bp and 549–550 bp T-RFs sizes obtained with *Msp*I restriction enzyme and the 359–360 bp T-RF size obtained with *Hha*I restriction enzyme as correlation coefficient indicated in the discriminant analysis. Microbial communities on the left of Axis 1 clustered mainly by the 66 bp, 129–130 bp, 163–164 bp and 428–429 bp T-RFs sizes obtained with *Msp*I restriction enzyme. Microbial communities on the top of Axis 2 clustered by the 132 bp, 139–140 bp and 158–159 bp obtained with *Msp*I restriction enzyme and the 359–360 bp T-RFs sizes obtained with *Hha*I restriction enzyme. Microbial communities on the bottom of Axis 2 clustered by the 494–495 bp T-RF size obtained with *Msp*I restriction enzyme. These T-RFs sizes, which were pointed out as mainly important in the discriminant analysis, had different relative abundance in the rhizosphere of wheat plants under the evaluated treatments at both phenological stages (Fig. 4). The discriminant analysis showed that the microbial communities clustered by the inoculation and fertilization treatments applied on these plants only at the grain-filling stage of the crop (Fig. 3). However, neither the

Table 2 Most probable number (MPN) of diazotrophic bacteria in the rhizosphere of wheat plants at two phenological stages

Phenological stage	Treatments			Means
	Control	Inoculated	Fertilized	
	(log MPN g^{-1} root) ± standard deviation			
Jointing	6.14 ± 0.54 a	6.16 ± 0.29 a	5.87 ± 0.39 a	6.06 ± 0.42 *b*
Grain-filling	5.79 ± 0.64 a	5.17 ± 0.62 a	5.27 ± 0.75 a	5.41 ± 0.70 *a*

Different italic letters indicate significant differences with Tukey's test ($P \leq 0.05$) between phenological stages with $n = 24$

Different normal letters indicate significant differences with Tukey's test ($P \leq 0.05$) between treatments at each phenological stage with $n = 12$

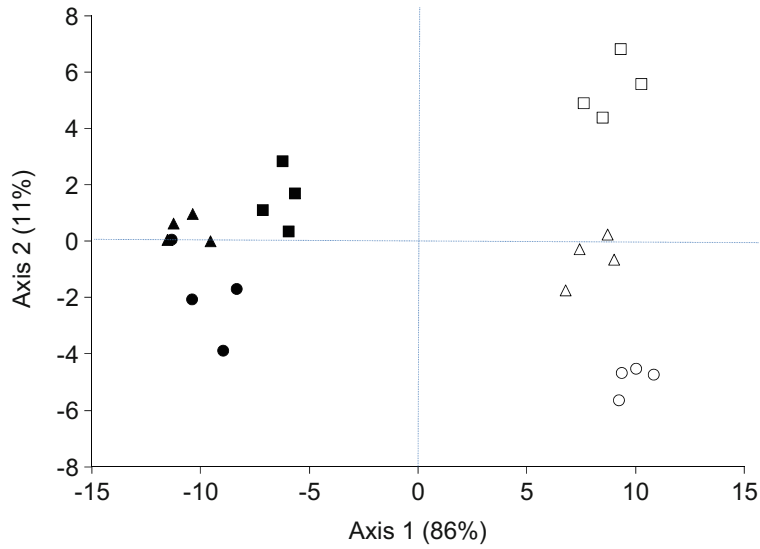

Fig. 1 Discriminant analysis of the CLPP of bacterial communities on roots of wheat under three different treatments at jointing (white symbols) and grain-filling (black symbols) stages ($n = 24$). Circles correspond to control plots, squares to fertilized plots, and triangles to inoculated plots. Data used for the analysis correspond to 48 h absorbance values. Total explained variance by each axis is in parenthesis

plant ontogeny nor the evaluated treatments modified the structural diversity evaluated with T-RFLP of microbial communities from rhizosphere of wheat plants (Table 3).

Discussion

Many authors demonstrated increases in wheat grain yield (Díaz-Zorita et al. 2015; García de Salamone 2012a; Naiman et al. 2009) in response to *A. brasilense* inoculation. Although in this work edaphoclimatic conditions were in the optimal range for the wheat production, grain yield and aerial biomass did not show increases due to N fertilization and inoculation with 40M and 42M *A. brasilense* strains compared to control plants (Table 1). It has been vastly discussed that the complex plant-microbe-environment interactions cause variations in the level of agronomic response to *A. brasilense* inoculation (Cassán and Díaz-Zorita 2016), including plant and bacteria genotypes (García de Salamone 2012a). At this regard, the two *A. brasilense* strains used in this work were isolated from maize (García de Salamone and Döbereiner 1996). Despite non agronomic response were observed in this wheat field experiment, they promoted

Table 3 Functional and structural diversity of rhizosphere microbial communities of wheat plants at two phenological stages

Phenological stage	Treatments			Means
	Control	Inoculated	Fertilized	
	Shannon index[a] ± standard deviation			
Jointing	2.87 ± 0.03 a	2.93 ± 0.05 a	2.88 ± 0.05 a	2.89 ± 0.05 *a*
Grain-filling	3.05 ± 0.02 a	3.06 ± 0.01 a	3.06 ± 0.02 a	3.06 ± 0.02 *b*
	Shannon index[b] ± standard deviation			
Jointing	2.84 ± 0.15 a	2.81 ± 0.07 a	2.66 ± 0.20 a	2.77 ± 0.16 *a*
Grain-filling	2.75 ± 0.18 a	2.83 ± 0.10 a	2.83 ± 0.12 a	2.80 ± 0.13 *a*

Different italic letters indicate significant differences with Tukey's test ($P \leq 0.05$) between phenological stages with $n = 24$

Different normal letters indicate significant differences with Tukey's test ($P \leq 0.05$) between treatments at each phenological stage with $n = 12$

[a] Shannon index calculated with CLPP data (functional diversity)

[b] Shannon index calculated with T-RFLP data (structural diversity)

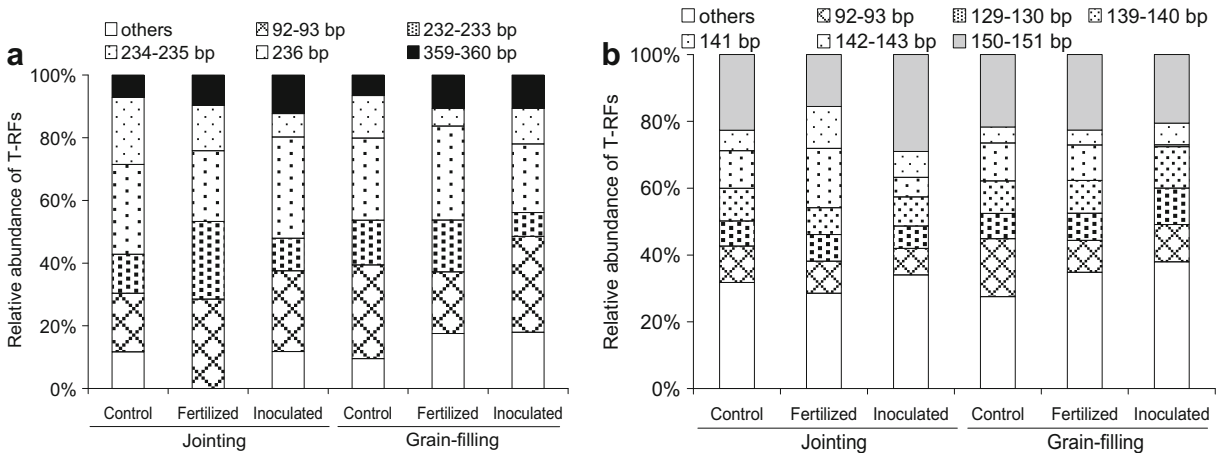

Fig. 2 Relative abundance of the most abundant terminal restriction fragment (T-RFs) sizes obtained after the digestion of the 16S rRNA amplified products from rhizosphere of wheat plants under the three treatments at two phenological stages ($n = 24$). A. Digestion was performed with Hha I restriction enzyme. The "others" group includes T-RFs sizes which signal percentage under 10% of the total signal and they are: 51–52 bp, 54 bp, 56 bp, 70 bp, 73 bp, 202 bp, 224 bp and 231 bp. B. Digestion was performed with *Msp*I restriction enzyme. The "others" group includes T-RFs sizes which signal percentage under 10% of the total signal and they are: 51–52 bp, 59–60 bp, 62 bp, 63–64 bp, 66 bp, 72 bp, 126–127 bp, 132 bp, 136–137 bp, 144 bp, 146–147 bp, 148 bp, 158–159 bp, 160–161 bp, 163–164 bp, 166 bp, 428–429 bp, 494–495 bp, 546–547 bp and 549–550 bp

grain yield and aerial biomass of maize (García de Salamone and Döbereiner 1996) and rice (García de Salamone et al. 2010).

It is important to distinguish between this lack of statistical response and the economic advantage of increasing grain yield by the inoculation (García de Salamone 2012a; Naiman et al. 2009). While the use of inoculants contributes in small amount of money to the cost of crops, any beneficial increase of grain yield is gratefully accepted by the farmers. In addition, any increase in aerial biomass involves higher plant residues after harvest, which will be added to the soil to maintain organic matter pool. Besides of the greater amount and a higher quality of the stubble to the humification process (Sisti et al. 2004), it could allow the accumulation of soil organic matter in order to increase soil quality and agricultural sustainability (Grandy et al. 2006).

The structural diversity of microbial communities refers to its genetic composition, while the functional diversity refers to ecosystem functions of these

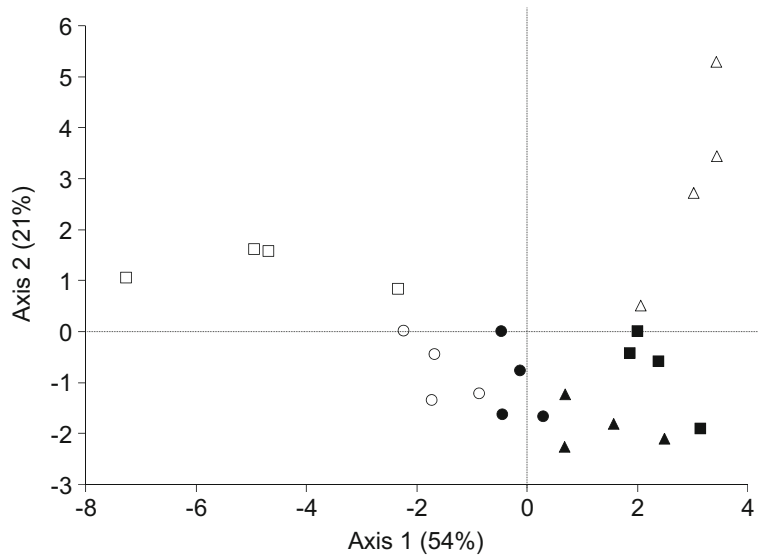

Fig. 3 Discriminant analysis of the T-RFLP of rhizospheric soil of wheat under three different treatments at jointing (white symbols) and grain-filling (black symbols) stages ($n = 24$). Circles correspond to control plots, squares to fertilized plots, and triangles to inoculated plots. Data used for the analysis correspond to restriction fragments obtained with *Msp*I and *Hha*I enzymes. Total explained variance by each axis is in parenthesis

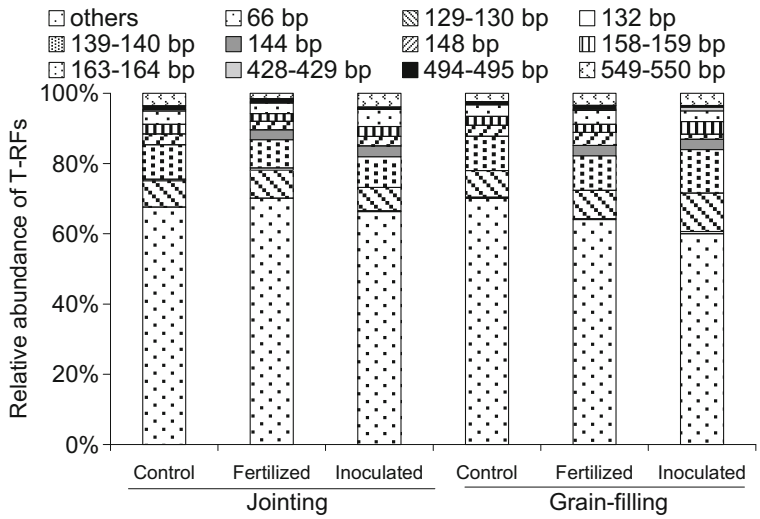

Fig. 4 Relative abundance of the terminal restriction fragment (T-RFs) sizes which were pointed out as mainly important in the discriminant analysis of T-RFLP data as correlation coefficient indicated. By contrast, the "others" group includes T-RFs sizes which pointed out as less important in the discriminant analysis of T-RFLP data and they are: 51–52 bp, 59–60 bp, 62 bp, 63–64 bp, 72 bp, 92–93 bp, 126–127 bp, 136–137 bp, 141 bp, 142–143 bp, 146–147 bp, 150–151 bp, 160–161 bp, 166 bp and 546–547 bp. The T-RFs sizes were obtained after the digestion with *Msp*I restriction enzyme of the 16S rRNA amplified products from rhizosphere of wheat plants under the three treatments in both phenological stages ($n = 24$)

microbial communities. The relationship between structural diversity and functional diversity is still unknown but under persistently assessment (Bastida et al. 2008; Mendes et al. 2014). Thus, this is the greatest challenge of soil microbial ecology (Torsvik and Øvreås 2002). Besides, studies on aboveground-belowground biota interactions and biodiversity can be used to evaluate effects of human-induced environmental changes (Wardle et al. 2004). Interestingly, in this work, agricultural treatments did not affect aboveground plant biomass and grain yield but, by contrast, modified underground microbial communities.

The functional redundancy phenomenon refers to the 'ability of one microbial taxon to carry out a process at the same rate as another under the same environmental conditions' (Allison and Martiny 2008). Changes in structural diversity of microbial communities could not affect ecosystem functions due to the functional redundancy (Allison and Martiny 2008; Bossio et al. 2005), particularly in disturbed ecosystems such as agricultural (Mendes et al. 2015). Despite this, in this work both CLPP and T-RFLP analyses showed that *A. brasilense* inoculation and N fertilization modified temporarily the structure and physiology of microbial communities of wheat rhizosphere (Figs. 1 and 3). These changes in structural and functional diversity were mainly observed at jointing stage. After a disturbance, such as inoculation and fertilization practices (van Bruggen and Semenov 2000), soil microbial communities could change (Kent and Triplett 2002; Minz and Ofek 2011) and return to a similar composition before the disturbance, depending on its resilience (Allison and Martiny 2008). In this regard, some authors demonstrated that repeated inoculations of biocontrol bacteria did not modify significantly the microbial communities (Steddom et al. 2002). On the contrary, periodic soil tillage modified them in relation to microbial communities from soil under low tillage system, which had a similar composition than original forest soil (Peixoto et al. 2006).

Despite the fact that carbon sources into the microplates and culture conditions do not represent the same conditions as in the soils (Preston-Mafham et al. 2002), the CLPP provides information about potential functionality of microbial communities (Mills and Garland 2002). The CLPP technique was used to differentiate microbial communities from different treatments (Di Salvo and García de Salamone 2012; Lupwayi et al. 1998; Semmartin et al. 2010). Inoculation with *A. brasilense* also modified the CLPP of the culturable microbial communities associated with rice (García de Salamone et al. 2010, 2012) and wheat (Naiman et al. 2009). In this work, both fertilization and inoculation treatments modified the physiological profiles of rhizobacteria communities of wheat plants at jointing

stage but these differences were not observed at grain-filling stage (Fig. 1). Although the physiological profiles of the *A. brasilense* strains used in this work were characterized *in vitro* conditions (Di Salvo et al. 2014), they could not be directly used to explain the differences in the CLPP profiles observed in this work because the whole rhizosphere microbial community has being profiled at field conditions. Interestingly, similarly as the analysis of CLPP profiles, the analysis of structural profiles showed that both fertilization and inoculation treatments modified the structural profiles of rhizobacteria communities of wheat plants at jointing stage but these differences were not observed at grain-filling stage (Fig. 3). In addition, some authors evaluated microbial diversity using *H* index calculated with data of CLPP (Di Salvo and García de Salamone 2012; Gómez et al. 2004) and 16S–rRNA gene profiles obtained with T-RFLP (Sawamura et al. 2010). In this work, *H* index from CLPP data represents functional diversity while *H* index from T-RFLP data represents structural diversity. Interestingly, only the former showed differences between phenological stages of wheat plants. This result could be related with observed changes in microbial diversity during the plant growth cycle due to differences in quantity and quality of roots exudates reported by several authors (Herschkovitz et al. 2005; Houlden et al. 2008; Philippot et al. 2013).

The T-RFLP technique was pointed out as one of the most robust and reliable method used to characterize structural diversity of microbial communities (Smalla et al. 2007). Thus, several authors used this technique to evaluate diversity of microbial communities (Ferrando et al. 2012; Sawamura et al. 2010). Additionally, T-RFLP allows a potential assignment of the T-RF size (Dunbar et al. 2001). This assignment is potential due to a particular T-RF size could come from many different bacterial species (Smalla et al. 2007). Besides, a T-RF size can be assigned to certain microbial genus if it was isolated and identified previously, and if its sequence was deposited in the database to be used later for *in silico* digestion and subsequent comparison with the sequence to be identified. Thus, unknown genera and not available on database cannot be compared with sequences to be identified and could not to be assigned. Despite these limitations, in this work some of the T-RFs sizes obtained with both restriction enzymes (Figs. 2 and 4) were compared with *in silico* digestions of sequences from database and assigned to some known microbial genera, similarly as Sawamura et al. (2010).

The 359–360 bp T-RF size obtained with *Hha*I restriction enzyme presented high relative abundance (Fig. 2.A) and it was considered as mainly important in the discriminant analysis to distinguish structural profiles of rhizobacteria communities of wheat plants. Most sequences with 359–360 bp T-RF size (Fig. 2.A) could correspond to the genus *Streptomonospora*, an halophilic actinomycete isolated from soils (Li et al. 2003) but not in the rhizosphere of crop plants. The 92–93 bp, 141 bp, 129–130 bp, 142–143 bp and 150–151 bp T-RF size obtained with *Msp*I restriction enzyme were some of the most abundant in the profiles of rhizobacteria communities of wheat plants (Fig. 2.B). Most sequences with 92–93 bp T-RF size (Fig. 2.B) could correspond to the genus *Bacteroides*, which was observed in the rhizosphere of some crop plants, such as soybean (Xu et al. 2009).

Most sequences with 141 bp T-RF size (Fig. 2.B) could correspond to the PGPR genus *Burkholderia*, which is frequently found in the rhizosphere of some crop plants, such as maize (Di Cello et al. 1997), rice (Gillis et al. 1995) and tomato (Caballero-Mellado et al. 2007). Most sequences with 129–130 bp T-RF size (Fig. 2.B) could correspond to the *Paracoccus*, *Thiothrix*, *Anaeromyxobacter* and *Gluconacetobacter* genera. All of them were found in the rhizosphere of some crop plants, such as rice (Lu et al. 2006) and coffee (Fuentes-Ramírez et al. 2001). Most sequences with 142–143 bp T-RF size (Fig. 2.B) could correspond to the genus *Nocardioides*, which was found in wheat rhizosphere (Conn and Franco 2004). Finally, the 150–151 bp T-RF size had in average 22% of relative abundance and it was one of the most abundant T-RFs sizes (Fig. 2.B). Most sequences with 150–151 bp T-RF size (Fig. 2.B) could correspond to the *Paenibacillus*, *Erythrobacter*, *Nostoc*, *Methylobacterium* and *Azospirillum* genera. Some authors showed that *Paenibacillus* is present in wheat rhizosphere (Germida and Siciliano 2001). The genus *Erytrhobacter* was isolated from marine habitats (Koblížek et al. 2003) but until now, it has not been found in the rhizosphere. The genus *Methylobacterium* is ubiquitous, and usually colonizes wheat rhizosphere (Sanguin et al. 2009). In experimental trials, *Nostoc* spp. has also shown to colonize the rhizosphere of wheat (Karthikeyan et al. 2007) after inoculation.

Results regarding to relative abundance of T-RFs sizes are often shown in the literature in the same way as were shown in this work (Fig. 2). However, in this work the discriminant analysis of T-RFLP data (Fig. 3)

was also included because it is more useful to distinguish differences between treatments or phenological stage than the relative abundance of the most abundant T-RFs sizes (Fig.2). Even the relative abundance of the T-RFs sizes, which were pointed out as mainly important in the discriminant analysis, showed some differences between treatments (Fig. 4). In this regard, the 144 bp T-RF size was not one of the most abundant T-RFs sizes obtained with *Msp*I restriction enzyme (Fig. 2.B) but was pointed out as mainly important in the discriminant analysis to distinguish structural profiles of rhizobacteria communities of wheat plants. This T-RF size was present in treated plants but not in control plants (Fig. 4). After *in silico* digestion of sequences from database with the *Msp*I restriction enzyme, most sequences with 144 bp T-RF size could correspond to *Bacillus* and *Pseudomonas* genera, which are frequently isolated from soils and vastly recognized as PGPR (Cummings 2009; Gamalero and Glick 2011; García de Salamone et al. 2012). Due to the inoculation and N fertilization treatments increased the relative abundance of 144 bp T-RF size (Fig. 4), which could be assigned to *Bacillus* and *Pseudomonas* genera., it seems that they could be used as co-inoculants for wheat, even under interaction with N fertilizers. In this regard, there are few references about co-inoculation and most of these papers are not related with these three PGPR or not related with wheat. One of them, showed interactions between *A. brasilense* and *P. fluorescens* co-inoculation and rice cultivars (García de Salamone et al. 2012). This suggests that more studies are needed to evaluate crop responses to co-inoculation of *A. brasilense* strains and other bacteria.

It is interesting to analyze that, on one hand, the 150–151 bp (*Msp*I) T-RF size, which could correspond to *Azospirillum* spp., was present in the rhizosphere of both control and treated wheat plants (Fig. 2.B). On the other hand, the three evaluated treatments did not show differences in the MPN of diazotrophic rhizobacteria associated with wheat plants (Table 2). Thus, both results could be considered as consistent and related between them, although, 150–151 bp (*Msp*I) T-RF size is not exclusive for diazotrophic rhizobacteria like *Azospirillum* spp., and in the culture media used to determine the MPN could be growing diazotrophic bacteria with T-RF other than 150–151 bp (*Msp*I). In this regard, other authors used the T-RFLP technique to show that *A. brasilense* was present in the rhizosphere soil samples from control and inoculated wheat plants although as it was pointed out by Kazi et al. (2016): 'inferences of identity from T-RFLP profiles must be interpreted cautiously as multiple species can have T-RFs of identical length'.

The semisolid Nfb medium was formulated in order to isolate and characterize microaerophilic nitrogen-fixing *A. brasilense* strains due to its typical growth (Döbereiner 1998). Recently, it was demonstrated that other nitrogen-fixing bacteria different from *A. brasilense* could grow in this medium (Di Salvo et al. 2014).

According to what was previously observed in inoculation trials of rice (García de Salamone et al. 2010; Pedraza et al. 2009) and maize (Abril et al. 2006; Casaretto and Labandera 2008), in this work it was expected that MPN of diazotrophic rhizobacteria from inoculated plants was higher than the others, but no significant differences were observed (Table 2). In this regard, some authors have demonstrated that not always differences in the MPN of diazotrophic rhizobacteria can be detected in inoculation field assays (Abril et al. 2006; García de Salamone et al. 2012) due to the fact that other species different from *A. brasilense* or even native strains of this bacteria could grow in the culture medium. Despite the fact that MPN of diazotrophic rhizobacteria has not been modified by the evaluated treatments, the MPN of this functional group at joining stage was higher than the MPN at grain-filling stage. In this regard, other authors showed that rhizosphere colonization by this functional group of bacteria decreased during maize and rice growth using the same methodology as in this work (García de Salamone et al. 2010; Roesch et al. 2006).

In conclusion, we demonstrated that plant ontogeny mainly modified both physiology and genetic structure of rhizosphere microbial communities. These differences were observed with the discriminant analysis, while only functional diversity (H index form CLPP profiles) showed differences between ontogenetic stages. In addition, plant ontogeny modified the number of culturable microaerophilic diazotrophic rhizobacteria. In opposition to our hypothesis, in this work, the inoculation with 40M and 42M *A. brasilense* strains and the chemical fertilization with 46 kg N ha^{-1} N did not modify the agronomic response of the wheat crop but modified both the genetic and functional characteristics of microbial communities associated with the rhizosphere of wheat plants under field conditions. Changes were observed only at jointing stage but

not at grain-filling stage. Our results demonstrate how different management decisions can change plant-microorganism relationships. One of the most important limitations to link the aboveground to belowground ecology has been the lack of information on soil microbiology (Hooper et al. 2000). In this regard, this work demonstrates that wheat crop could not show differences between some agricultural treatments but, by contrast, under the soil surface microbial communities could show them. The results of this work will help to scientist, professionals and farmers to obtain a better understanding of microbial ecology in order to improve yields and to obtain sustainable production under the new green revolution paradigm.

Acknowledgements This work was partially supported by FONCYT 2008 PICT 1864 from the MINCyT, UBACyT project 20020090100255, Universidad de Buenos Aires (UBA) in Argentina. The authors received financial support from the collaborative projects PROMAI UBA and AUGM for travel expenses of L.P.D.S., L.F. and A.F.S. We are grateful to the agronomist Patricio Perdoménico and personal of "La Aurora", Villa Moll, Buenos Aires, Argentina. We are also grateful to editors and anonymous reviewers for their comments and suggestions. We would like to dedicate this work to the memory of Dr. Katia RS Teixeira, Brazilian researcher of the EMBRAPA, Rio de Janeiro, Brazil, who always will be in our hearts.

References

Abril A, Biasutti C, Maich R, Dubbini L, Noe L (2006) Inoculación con *Azospirillum* spp. en la región semiárida-central de la Argentina: factores que afectan la colonización rizosférica. Ciencia Suelo 24:11–19

Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A 105:11512–11519

Bashan Y, de-Bashan LE (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. Adv Agron 108:77–136

Bastida F, Zsolnay A, Hernández T, García C (2008) Past, present and future of soil quality indices: A biological perspective. Geoderma 147:159–171

Bossio DA, Girvan MS, Verchot L, Bullimore J, Borelli T, Albrecht A, Scow KM, Ball AS, Pretty JN, Osborn AM (2005) Soil microbial community response to land use change in an agricultural landscape of Western Kenya. Microb Ecol 49:50–62

van Bruggen AHC, Semenov AM (2000) In search of biological indicators for soil health and disease suppression. App Soil Ecol 15:13–24

Caballero-Mellado J, Onofre-Lemus J, Estrada-de los Santos P, Martínez-Aguilar L (2007) The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. Appl Environ Microbiol 73:5308–5319

Casanovas EM, Fasciglione G, Barassi CA (2015) *Azospirillum* spp. and related PGPRs inocula use in intensive agriculture. In: Cassán FD, Okon Y, Creus CM (eds) Handbook for *Azospirillum*: Technical Issues and Protocols. Springer, Berlin, pp 447–468

Casaretto E, Labandera C (2008) Respuesta de maíz a la inoculación con *Azospirillum*. In: Cassán FC, García de Salamone IE (eds) *Azospirillum* sp.: cell physiology, plant interactions and agronomic research in Argentina, Asociación Argentina de Microbiología, Buenos Aires, Argentina, pp. 261–268

Cassán FD, Díaz-Zorita M (2016) *Azospirillum* sp. in current agriculture: From the laboratory to the field. Soil Biol Bioch 103:117–130

Cassán FD, Okon Y, Creus MC (2015) Handbook for *Azospirillum*: Technical Issues and Protocols. Springer, Berlin

Conn VM, Franco CMM (2004) Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA. Appl Environ Microbiol 70:1787–1794

Cummings SP (2009) The application of plant growth promoting rhizobacteria (PGPR) in low input and organic cultivation of graminaceous crops; potential and problems. Environ Biot 5: 43–50

Den Herder G, Van Isterdael G, Beeckman T, De Smet I (2010) The roots of a new green revolution. Trends Plant Sci 15: 600–607

Díaz-Zorita M, Fernández-Canigia MV, Bravo OA, Berger A, Satorre EH (2015) Field evaluation of extensive crops inoculated with *Azospirillum* sp. In: Cassán FD, Okon Y, Creus CM (eds) Handbook for *Azospirillum*: Technical Issues and Protocols. Springer, Berlin, pp 435–445

Di Cello F, Bevivino A, Chiarini L, Fani R, Paffetti D, Tabacchioni S, Dalmastri C (1997) Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Appl Environ Microbiol 63: 4485–4493

Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW (2011) InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina

Di Salvo LP, García de Salamone IE (2012) Evaluation of soil-microbial communities by their CLPP. Standardization of a laboratory technique to replace commercial available microplates. Austral Ecol 22:129–136

Di Salvo LP, Silva E, Teixeira KR, Esquivel-Cote R, Pereyra MA, García de Salamone IE (2014) Physiological and biochemical characterization of *Azospirillum brasilense* strains commonly used as plant growth-promoting rhizobacteria. J Basic Microbiol 54:1310–1321

Döbereiner J (1998) Isolation and identification of aerobic nitrogen-fixing bacteria from soil and plants. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, California, pp 134–141

Döbereiner J, Pedrosa F (1987) Nitrogen-fixing bacteria in non-leguminous crop plant. Spring, Michigan

Dunbar J, Ticknor LO, Kuske CR (2001) Phylogenetic specificity and reproducibility and new method for analysis of terminal

restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Environ Microbiol 67:190–197

FAOSTAT (2012) Food and Agricultural commodities production. http://faostat.fao.org/site/339/default.aspx

Ferrando L, Fernández Mañay J, Fernández Scavino A (2012) Molecular and culture-dependent analyses revealed similarities in the endophytic bacterial community composition of leaves from three rice (*Oryza sativa*) varieties. FEMS Microbiol Ecol 80:696–708

Fuentes-Ramírez LE, Bustillos-Cristales R, Tapia-Hernández A, Jiménez-Salgado T, Wang ET, Martínez-Romero E, Caballero-Mellado J (2001) Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int J Syst Evol Microbiol 51:1305–1314

Gamalero E, Glick BR (2011) Mechanisms used by plant growth-promoting bacteria. In: Maheshwari DK (ed) Bacteria in agrobiology: Plant nutrient management. Springer, Berlin, pp 17–46

García de Salamone IE (2012a) Use of soil microorganisms to improve plant growth and ecosystem sustainability. In: Caliskan M (ed) The molecular basis of plant genetic diversity. INTECH, Rijeka, Croatia, pp. 233–258. Open access: http://www.intechopen.com/articles/show/title/use-of-soil-microorganisms-to-improve-plant-growth-and-ecosystem-sustainability

García de Salamone IE (2012b) Microorganismos promotores del crecimiento vegetal. Informaciones Agronómicas de Hispanoamérica 5:12–16. http://www.ipni.net/publication/ia-lahp.nsf/0/C101526851953B09852579DD006C1A17/$FILE/3%20Art.pdf

García de Salamone IE, Döbereiner J (1996) Maize genotype effects on the response to *Azospirillum* inoculation. Biol Fert Soils 21:193–196

García de Salamone IE, Di Salvo LP, Escobar Ortega JS, Boa Sorte MP, Urquiaga S, Dos Santos Teixeira KR (2010) Field response of rice paddy crop to inoculation with *Azospirillum*: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants. Plant Soil 336:351–362

García de Salamone IE, Funes JM, Di Salvo LP, Escobar Ortega JS, D'Auria F, Ferrando L, Fernandez Scavino A (2012) Inoculation of paddy rice with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact of plant genotypes on the rhizosphere microbial communities and field crop production. Appl Soil Ecol 61:196–204

Germida JJ, Siciliano SD (2001) Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. Biol Fert Soils 33:410–415

Gewin V (2010) An underground revolution. Nature 466:552–553

Gillis M, Tran Van V, Bardin R, Goor M, Hebbar P, Willems A, Segers P, Kersters K, Heulin T, Fernandez MP (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnarniensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. Int J Syst Bacteriol 45:274–289

Gil-Sotres F, Trasar-Cepeda C, Leiros MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Gómez E, Garland J, Conti M (2004) Reproducibility in the response of soil bacterial community-level physiological profiles from a land use intensification gradient. Appl Soil Ecol 26:21–30

Grandy AS, Robertson GP, Thelen KD (2006) Do productivity and environmental trade-offs justify periodically cultivating no-till cropping systems? Agron J 98:1377–1383

Herschkovitz Y, Lerner A, Davidov Y, Rothballer M, Hartmann A, Okon Y, Jurkevitch E (2005) Inoculation with the plant-growth-promoting rhizobacterium *Azospirillum brasilense* causes little disturbance in the rhizosphere and rhizoplane of maize (*Zea mays*). Microb Ecol 50:277–288

Hooper DU, Bignell DE, Brown VK, Brussard L, Dangerfield JM, Wall DH, Wardle DA, Coleman DC, Giller KE, Lavelle P, Van der Putten WH, De Ruiter PC, Rusek J, Silver WL, Tiedje JM, Wolters V (2000) Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: patterns, mechanisms, and feedbacks. Bioscience 50:1049–1061

Houlden A, Timms-Wilson TM, Day MJ, Bailey MJ (2008) Influence of plant development stage on microbial community structure and activity in the rhizosphere of three field crops. FEMS Microbiol Ecol 65:193–201

Hungria M, Campo RJ, Souza EM, Pedrosa FO (2010) Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. Plant Soil 331:413–425

Karthikeyan N, Prasanna R, Nain L, Kaushik BD (2007) Evaluating the potential of plant growth promoting cyanobacteria as inoculants for wheat. Eur J Soil Biol 43:23–30

Kazi N, Deaker R, Wilson N, Muhammad K, Trethowan R (2016) The response of wheat genotypes to inoculation with *Azospirillum brasilense* in the field. Field Crop Res 196:368–378

Kent AD, Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. Ann Rev Microbiol 56:211–236

Kobližek M, Béjà O, Bidigare RR, Cheristensen S, Benitez-Nelson B, Vetriani C, Kolber MK, Falkowski PG, Kolber ZS (2003) Isolation and characterization of *Erythrobacter* sp. strains from the upper ocean. Arch Microbiol 180:327–338

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Meth 58:169–188

Li WJ, Xu P, Zhang LP, Tang SK, Cui XL, Mao PH, Xu LH, Schumann P, Stackebrandt E, Jiang CL (2003) *Streptomonospora alba* sp. nov., a novel halophilic actinomycete, and emended description of the *genus Streptomonospora* Cui *et al.* 2001. Int J Syst Evol Microbiol 53:1421–1425

Lu Y, Rosencrantz K, Liesack W, Conrad R (2006) Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. Environ Microbiol 8:1351–1360

Lupwayi NZ, Rice WA, Clayton GW (1998) Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biol Biochem 30:1733–1741

de Man JC (1983) MPN tables, corrected. Eur J Appl Microbiol Biotech 17:301–305

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. App Environ Microbiol 64:795–799

Mendes LW, Kuramae EE, Navarrete AA, van Veen JA, Tsai SM (2014) Taxonomical and functional microbial community selection in soybean rhizosphere. ISME J 8:1577–1587

Mendes LW, Tsai SM, Navarrete AA, de Hollander M, van Veen JA, Kuramae EE (2015) Soil-borne microbiome: linking diversity to function. Microb Ecol 70:255–265

Miethling R, Wieland G, Backhaus H, Tebbe CC (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. Microb Ecol 41:43–56

Mills AL, Garland JL (2002) Application of physiological profiles to assess community properties. In: Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stenzenback LD (eds) Manual of environmental microbiology. ASM Press, Washington DC, pp 135–146

Minz D, Ofek M (2011) Rhizosphere microorganisms. In: Rosenberg E, Gophna U (eds) Beneficial microorganisms in multicellular life forms. Springer, Verlag, pp 105–121

Naiman AD, Latrónico A, García de Salamone IE (2009) Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: impact on the production and culturable rhizosphere microflora. Eur J Soil Biol 45:44–51

Pedraza RO, Bellone CH, Carrizo de Bellone S, Fernandes Boa Sorte PM, Teixeira KRS (2009) *Azospirillum* inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop. Eur J Soil Biol 45:36–43

Peixoto RS, Coutinho HLC, Madari B, Machado PLOA, Rumjanek NG, Van Elsas JD, Seldin L, Rosado AS (2006) Soil aggregation and bacterial community structure as affected by tillage and cover cropping in the Brazilian Cerrados. Soil Till Res 90:16–28

Philippot L, Raaijmakers JM, Lemanceau P, Van der Putten W (2013) Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev 11:789–799

Preston-Mafham J, Boddy L, Randerson PF (2002) Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles - a critique. FEMS Microbiol Ecol 42:1–14

Roesch LFW, Olivares FL, Passaglia LMP, Selbach PA, Saccol de Sá EL, Camargo FAO (2006) Characterization of diazotrophic bacteria associated with maize: effect of plant genotype, ontogeny and nitrogen-supply. World J Microb Biot 22:967–974

Saleem M, Moe LA (2014) Multitrophic microbial interactions for eco- and agro-biotechnological processes: theory and practice. Trends Biotechnol 32:529–537

Sanguin H, Sarniguet A, Gazengel K, Moënne-Loccoz Y, Grundmann GL (2009) Rhizosphere bacterial communities associated with disease suppressiveness stages of take-all decline in wheat monoculture. New Phytol 184:694–707

Sawamura H, Yamada M, Endo K, Soda S, Ishigaki T, Ike M (2010) Characterization of microorganisms at different landfill depths using carbon-utilization patterns and 16S rRNA gene based T-RFLP. J Biosci Bioeng 109:130–137

Semmartin M, Di Bella C, García de Salamone IE (2010) Grazing-induced changes in plant species composition affect plant and soil properties of grassland mesocosms. Plant Soil 328:471–481

Shyu C, Soule T, Bent SJ, Foster JA, Forney LJ (2007) MiCA: A Web-Based Tool for the Analysis of Microbial Communities Based on Terminal-Restriction Fragment Length Polymorphisms of 16S and 18S rRNA Genes. J Microb Ecol 53:562–570

Sisti CPJ, Santos HP, Kochhann R, Alves BJR, Urquiaga S, Boddey RM (2004) Change in carbon and nitrogen stocks in soil under 13 years of conventional and zero tillage in southern Brazil. Soil Till Res 76:39–58

Smalla K, Oros-Sichler M, Milling A, Heuer H, Baumgarte S, Becker R, Neuber G, Kropf S, Ulrich A, Tebbe CC (2007) Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: Do the different methods provide similar results? J Microbiol Meth 69:470–479

Steddom K, Menge JA, Crowley D, Borneman J (2002) Effect of repetitive applications of the biocontrol bacterium *Pseudomonas putida* 06909-rif/nal on citrus soil microbial communities. Biol Control 92:857–862

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671–677

Torsvik V, Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240–245

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. Science 304:1629–1633

Xu Y, Wang G, Jin J, Liu J, Zhang Q, Liu X (2009) Bacterial communities in soybean rhizosphere in response to soil type, soybean genotype, and their growth stage. Soil Biol Biochem 41:919–925

Xue D, Yao HY, Ge DY, Huang CY (2008) Soil microbial community structure in diverse land use systems: a comparative study using Biolog, DGGE, and PLFA analyses. Pedosphere 18:653–663