Journal of Basic Microbiology
Environment · Health · Techniques

Full Paper

Physiological and biochemical characterization of *Azospirillum brasilense* strains commonly used as plant growth-promoting rhizobacteria

Luciana P. Di Salvo[1], Esdras Silva[2], Kátia R.S. Teixeira[2], Rosalba Esquivel Cote[3], M. Alejandra Pereyra[4] and Inés E. García de Salamone[1]

[1] Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina
[2] Embrapa Agrobiologia, Seropédica, Brazil
[3] Departamento de Biología, Laboratorio de Microbiología Experimental, México DF, México
[4] Laboratorio de Bioquímica Vegetal y Microbiana, Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Balcarce, Argentina

Azospirillum is a plant growth-promoting rhizobacteria (PGPR) genus vastly studied and utilized as agriculture inoculants. Isolation of new strains under different environmental conditions allows the access to the genetic diversity and improves the success of inoculation procedures. Historically, the isolation of this genus has been performed by the use of some traditional culture media. In this work we characterized the physiology and biochemistry of five different *A. brasilense* strains, commonly used as cereal inoculants. The aim of this work is to contribute to pose into revision some concepts concerning the most used protocols to isolate and characterize this bacterium. We characterized their growth in different traditional and non-traditional culture media, evaluated some PGPR mechanisms and characterized their profiles of fatty acid methyl esters and carbon-source utilization. This work shows, for the first time, differences in both profiles, and ACC deaminase activity of *A. brasilense* strains. Also, we show unexpected results obtained in some of the evaluated culture media. Results obtained here and an exhaustive knowledge revision revealed that it is not appropriate to conclude about bacterial species without analyzing several strains. Also, it is necessary to continue developing studies and laboratory techniques to improve the isolation and characterization protocols.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: Agriculture sustainability / Biological nitrogen fixation / Fatty acid methyl esters analysis / Plant growth promotion mechanisms PGPR / Physiological profiles

Received: February 14, 2014; accepted: May 26, 2014

DOI 10.1002/jobm.201400135

Introduction

Azospirillum is a plant growth-promoting rhizobacteria (PGPR) which produce direct and indirect beneficial effects on several crops [1]. The level of the *Azospirillum* inoculation response can be defined by the genetic characteristics of both bacteria and host plant [2]. It is necessary to obtain new isolates under different environmental conditions to guarantee access to the genetic diversity of *Azospirillum* spp. and, consequently, improve the success of inoculation procedure and plant growth promotion to agriculture sustainability. Historically, the isolation of strains of the genus *Azospirillum*, mainly *A. brasilense* and *A. lipoferum*, has been performed by the use of semisolid culture media, such as Nfb medium [3]. By aerotaxis, bacteria move to the specific place in the medium where the rate of oxygen diffusion and bacterial respiration allows nitrogenase enzyme to fix N_2 without the inhibitory effect of oxygen. Then, bacteria

Correspondence: Dr. Inés E. García de Salamone, Cátedra de Microbiología Agrícola, FAUBA, Avenida San Martín 4453, CABA, (C1417DSE), Argentina
E-mail: igarcia@agro.uba.ar
Phone/Fax: (54)11-4524-8061

form a typical sub-superficial veil-like pellicle growth [4]. Rodríguez-Cáceres [5] developed a different solid culture medium with the addition of Congo red dye. In this medium, referred as RC, colonies are described that they should be scarlet red, wrinkled, and dry. Despite of the carcinogenic action of Congo red and its inhibition effect on some bacterial species growth, some authors have pointed out that the RC medium had been very useful for obtaining new *Azospirillum* isolates [6].

Physiological characterization of the genus *Azospirillum* has been extensively considered in literature [4, 7, 8]. *Azospirillum* N_2 fixation is inhibited by the presence of oxygen [8]. Thus, it has been described that *Azospirillum* cannot grow in solid media without a nitrogen (N) source due to the lack of mechanisms of nitrogenase enzyme protection against oxygen [7]. Besides, it was pointed out that *A. brasilense* cannot grow under microaerophilic conditions in N-free media when sucrose or glucose is the sole carbon source (CS) [4, 8]. In addition, growth was not observed in presence of sucrose or glucose as CS when an N source was supplemented to the media [9], but it had been reported that certain strains had shown ability to grow in media containing glucose [10].

Bacterial cellular fatty acid analysis by gas chromatography (GC) of the obtained fatty acid methyl esters (FAME) has been used for more than 50 years as a rapid and easy-to-use method for routine microbial identification [11]. This chemotaxonomic analysis is commonly given in the species description. It has been applied for the rapid differentiation of *Rhizobium* spp. [12] and *Bradyrhizobium* spp. [13] species, among others. Schenk and Werner [14] proposed that an analysis of cellular fatty acids might be an appropriate method for identifying new isolates of *Azospirillum* genus. The results showed similarities and differences in the kind of fatty acids occurring among species of the genus *Azospirillum*.

Molecular techniques, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) fingerprinting or pulsed-field gel electrophoresis, have been used to differentiate some strains of the genus *Azospirillum*. However, some strains showed similar molecular patterns and cannot be differentiated, depending on both technique and protocol used [15, 16]. Recently, diverse genomic approaches are available and provide more efficient tools for studying genomic diversity [17, 18]. However, when strains are considered, those approaches are still expensive and may not be used as the main tool of choice to unravel the diversity of physiological and biochemical traits of *A. brasilense* during the first steps of isolation and characterization.

Much of the background information was considered during isolation and characterization of *A. brasilense* as microaerophilic N_2 fixing bacteria from soil and roots samples. However, results obtained during the isolation showed several unexpected behaviors [19]. In this regard, we performed a physiological and biochemical characterization of five *A. brasilense* strains. The aim of this study was to contribute to pose into revision some concepts concerning the most used protocols to isolate and characterize microaerophilic N_2-fixing bacteria of the genus *Azospirillum*, especially *A. brasilense*. The five strains evaluated in this work have already shown ability to promote growth of several crops under controlled and field conditions, and they are frequently used as plant inoculants [2, 20, 21]. In order to compare and characterize them, we described their growth features in traditional and non-traditional media. Also, we determined both siderophore and indolic compounds production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and phosphate solubilization. All these plant growth promoting mechanisms were analyzed under *in vitro* conditions. Furthermore, we have also characterized their profiles of FAME and CS utilization.

Materials and methods

Strains of *Azospirillum brasilense* and growth conditions

Five strains of *A. brasilense* were used in this study. The Sp7 (ATCC 29145; GenBank accession no. X79739), Cd (ATCC 29710; GenBank accession no. DQ438999), and Az39 (Agriculture Collection Laboratory; IMYZA-INTA, Argentina; GenBank accession no. JQ844453) strains were used as reference strains. The other two, 40M (Embrapa Agrobiology Diazothrophic Microbial Culture Collection BR11036, GenBank accession no. HM002661) and 42M (Embrapa Agrobiology Diazothrophic Microbial Culture Collection BR11038, GenBank accession no. HM002662), corresponded to *A. brasilense* strains isolated from maize and recently confirmed by 16S rRNA sequencing [2]. Evolutionary relationships of taxa based on 16S rDNA sequences of these *A. brasilense* strains and others was inferred using the Neighbor-Joining method and distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA5 [22] (Supporting Information Fig. S1).

Pure cultures of the five strains were inoculated on four traditional media to confirm their typical growth. There were semisolid Nfb medium (Nfb ss) [3], solid Nfb medium (Nfb) [3], Congo red medium (RC) [5], and TYG medium [23]. The incubation conditions of all media were 30 °C for 120 h. In cases where growth was observed, the

strains were inoculated three times in the same medium in order to confirm positive growth. When growth was not observed, inoculation was also repeated in order to confirm the incapability of the strain to grow under those conditions.

Growth in non-traditional semisolid media

Single colonies typical of the *A. brasilense* strains obtained in TYG medium were inoculated into vials with different semisolid media in order to evaluate if these strains can grow into media designed for other microorganisms, and fixing N_2 in microaerophilic conditions with non-traditional CS. After the incubation period, positive growth in each medium was considered when vials showed typical veil-like pellicle and pH change [3]. The semisolid evaluated media and their compositions are described as follows:

Semisolid LGI medium (LGI ss) for *A. amazonense* isolation [3]: Composition in $g\,L^{-1}$: K_2HPO_4 (0.2); KH_2PO_4 (0.6); $CaCl_2.2H_2O$ (0.002); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.002); $FeCl_3$ (0.01); Bromothymol Blue (BTB) solution (5.0 ml); sucrose (5.0); agar-agar (1.75); pH = 6.0.

BTB solution: 0.5% in 0.2 M KOH.

Semisolid JNfb medium (JNfb) for *Herbaspirillum* spp. isolation [3]: Composition in $g\,L^{-1}$: Malic acid (5.0); K_2HPO_4 (1.5); $CaCl_2.2H_2O$ (0.02); $MgSO_4.7H_2O$ (0.2); minor element solution (2.0 ml); vitamin solution (1 ml); Fe-EDTA (4.0 ml of 1.64% solution); BTB solution (2.0 ml); agar-agar (1.75); pH = 6.0.

Minor element solution (in $g\,L^{-1}$): $CuSO_4.5H_2O$ (0.4); $ZnSO_4.7H_2O$ (0.12); H_2BO_4 (1.4); $NaMoO_4.H_2O$ (1.0); $MnSO_4.H_2O$ (1.5).

Vitamin solution (%w/v): Biotin (0.01); Pyridoxal-HCl (0.02).

Semisolid LG salts with sucrose medium (LGsuc ss) was modified from LG medium for *Azotobacter* spp. and *Azomonas* spp. isolation [3]. Composition in $g\,L^{-1}$: Sucrose (20.0); K_2HPO_4 (0.05); KH_2PO_4 (0.15); $CaCl_2.2H_2O$ (0.01); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.002); $FeCl_2$ (0.01); BTB solution (2.0 ml); agar-agar (1.75); pH = 6.5.

Semisolid LG salts with glucose medium (LGglu ss) was modified from LG medium for *Azotobacter* spp. and *Azomonas* spp. isolation [3]. Composition in $g\,L^{-1}$: Glucose (20.0); K_2HPO_4 (0.05); KH_2PO_4 (0.15); $CaCl_2.2H_2O$ (0.01); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.002); $FeCl_2$ (0.01); BTB solution (2.0 ml); agar-agar (1.75); pH = 6.5.

Growth in non-traditional solid media

Single colonies typical of the *A. brasilense* strains obtained from TYG medium inoculated on two solid media without N source in order to test if N_2 fixation of these strains occurs in aerobic conditions, using others CS than traditional ones. The solid evaluated media and their compositions are described as follows:

Solid LG salts with sucrose medium (LGsuc) was modified from LG medium for *Azotobacter* spp. and *Azomonas* spp. isolation [3]. Composition in $g\,L^{-1}$: Sucrose (20.0); K_2HPO_4 (0.05); KH_2PO_4 (0.15); $CaCl_2.2H_2O$ (0.01); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.002); $FeCl_2$ (0.01); BTB solution (2.0 ml); agar-agar (15); pH = 6.7.

Solid LG salts with glucose medium (LGglu) was adapted from LG medium for *Azotobacter* spp. and *Azomonas* spp. isolation [3]. Composition in $g\,L^{-1}$: Glucose (20.0); K_2HPO_4 (0.05); KH_2PO_4 (0.15); $CaCl_2.2H_2O$ (0.01); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.002); $FeCl_2$ (0.01); BTB solution (2.0 ml); agar-agar (15); pH = 6.6.

Single colonies typical of the five strains obtained from Nfb medium were inoculated on different N-free media with different CS using UltraPure® Agarose (Invitrogen®) instead of agar-agar in order to discard any nutritional input from agar-agar. These media and their compositions are described as follows:

Semisolid Nfb with agarose medium (Nfb ss-agarose), solid Nfb with agarose medium (Nfb-agarose), and solid LG salts with glucose and agarose (LGglu-agarose). Same composition as Nfb ss, Nfb, and LGglu media, respectively, with agarose instead of agar-agar.

Solid LG salts without any CS and with agarose medium (LGagarose) was modified from LG medium for *Azotobacter* spp. and *Azomonas* spp. isolation [3]. Composition in $g\,L^{-1}$: K_2HPO_4 (0.05); KH_2PO_4 (0.15); $CaCl_2.2H_2O$ (0.01); $MgSO_4.7H_2O$ (0.2); $Na_2MoO_4.2H_2O$ (0.002); $FeCl_2$ (0.01); BTB solution (2.0 ml); agarose (15.0); pH = 6.5.

Plant growth promotion mechanisms

The plant growth promotion mechanisms phosphate solubilization, siderophore, and indolic compounds production and ACC deaminase activity of the five strains of *A. brasilense* were evaluated under *in vitro* conditions. Colonies of each one from TYG medium were collected and suspended in 0.5 ml of NaCl solution ($9\,g\,L^{-1}$ in water) and centrifuged at 12,000 rpm for 5 min. Supernatants were discarded. Cells were washed twice. After the last wash, strains were inoculated on two different media plates: Pikovskaya agar [24] and CAS agar [25]. Plates were incubated for 7 days at 30 °C. Halo formation indicates positive phosphate solubilization or positive siderophore production, respectively [24, 25].

Colonies of each strain from TYG medium were collected and suspended in 0.5 ml of NaCl solution ($9\,g\,L^{-1}$ in water) and centrifuged at 12,000 rpm for 5 min. Supernatants were discarded. Cells were washed twice. After the last wash, each strain were cultivated in flasks

with Nfb without BTB medium, and supplemented separately with ammonium chloride (1 g L^{-1}) or tryptophan (200 μg ml^{-1}) as N sources. Flasks were incubated for 4 days at 30 °C. After incubation, 1 ml of culture from each flask was centrifuged at 10,000 rpm for 10 min and 50 μl of supernatants were used to quantify production of indolic compounds in 96-well microplate [26]. Two indoleacetic acid (IAA) standard curves (0–10 μg) were performed in triplicates with a stock solution (1 mg IAA ml^{-1} methanol) and Nfb medium addition to complete 50 μl of total volume in each well. One of them included Nfb with ammonium chloride and the other one included Nfb with tryptophan. An aliquot of 200 μl of Salkowski's reagent (3 ml $FeCl_3$ 0.5M; 60 ml H_2SO_4 concentrated; and 100 ml distilled water) was added to each well. Microplate was incubated for 20 min at room temperature. After incubation, absorbance value was read with a microplate reader Multiskan EX® (Thermo, Vantaa, Finland) at 590 nm. Besides, counts of the number of cells of each strain were performed on TYG medium in order to make strains comparison.

ACC deaminase activity was evaluated by a protocol adapted from Hynes et al. [26]. Colonies of each strain from TYG medium were collected and suspended in 1 ml of NaCl solution (9 g L^{-1} in water) and centrifuged at 12,000 rpm for 5 min. Supernatants were discarded. Cells were washed twice. After the last wash, cells of each strain were suspended in 200 μl of NaCl solution (9 g L^{-1} in water) and cultivated in microplates with Nfb without BTB and ammonium chloride medium, and supplemented with 12 μl of ACC 5 mM solution as N source, using six replicates per each strain. Control wells without ACC addition were included. Microplates were incubated for 72 h at 30 °C. Before and after incubation, absorbance values were read with a microplate reader Multiskan EX® (Thermo) at 405 nm. After incubation, differences of absorbance values, with and without ACC, were standardized by the absorbance value before incubation to perform strain comparison.

Community-Level Physiological Profiles (CLPP)

The CLPP of *A. brasilense* strains were evaluated with 28 different CS in order to determine physiological differences among them. Colonies of each strain from TYG medium were collected and transferred to flasks with modified DYGS medium (Composition in g L^{-1}: glucose (2.0); malic acid (2.0); meat peptone (1.5); yeast extract (2.0); glutamic acid (1.5); K_2HPO_4 (0.5); $MgSO_4.7H_2O$ (0.5); pH = 6.8). The five strains were cultivated in triplicates. The flasks were incubated for 72 h at 30 °C. After incubation, 10-fold dilutions were performed. The 96-well microplates prepared in laboratory were inoculated with 50 μl from the 10^{-3} dilution. Each well also contained 100 μl of buffer medium, 50 μl of tetrazolium violet as redox dye indicator, and 50 μl of the respective CS [27]. The evaluated CS were arginine, glutamine, glycine, phenylalanine, proline, histidine, cellobiose, dextrose, maltose, rhamnose, xylose, fructose, glycerol, mannitol, lactic acid, malic acid, citric acid, oxalic acid, salicylic acid, benzoic acid, tween 20, putrescine, itaconic acid, arabinose, galactose, sucrose, sorbitol, and glyphosate. The pH range of final culture media in each well was 7.0 and 7.2. Microplates were incubated at 30 °C for 96 h. Absorbance values were taken every 24 h with a microplate reader Multiskan EX® (Thermo) at 590 nm. Data were used to obtain Average Well Color Development (AWCD) values. In this work, AWCD standardization of absorbance values was not included when significant differences between strains at 0 h reading were not observed [27]. Absorbance values higher than the AWCD were considered to calculate the number of CS used by each strain at every reading time. Absorbance values from 96 h of incubation were used to perform the multivariate statistical analyses.

Bacterial cellular FAME analysis by GC

The method proposed by Maas et al. [11] with slight modifications was used for the analysis of total cellular fatty acids. Colonies of each strain from RC medium were collected and suspended in 1 ml NaOH 1.2 N in 50% aqueous methanol and heated for 30 min at 100 °C. Extracts were then acidified to pH 2 with 6 N HCl, and treated with 1 ml of boron trichloride-methanol reagent (BCl_3-CH_3OH; Applied Science Laboratories, State College, Pennsylvania, USA). The mixture was heated at 85 °C for 10 min, cooled and extracted twice with hexane-diethyl ether (1:1). The organic phase, which contained the methyl esters, was transferred to a GC vial ready for analysis. The FAME were analyzed by GC using a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detector and a Chromatopac C-R6A integrator. The operating conditions were as follows: nitrogen carrier gas flow: 30 ml min^{-1}, injector and detector temperatures: 250 and 280 °C, respectively; initial and final column temperatures: 150 and 250 °C, respectively; temperature program rate 4 °C min^{-1}. One microliter samples were injected into SPB-5 30-m column (Supelco, Bellefonte, Pennsylvania, USA). Fatty acids were identified by co-chromatography with reference standards (Bacterial Acid Methyl Esters Mix, Catalog N1. 47080-U, Supelco).

Statistical analyses

CLPP and FAME profiles were analyzed using discriminant analyses. Number of CS used, indolic compounds

production, and CS utilization data were analyzed by ANOVA and Tukey's test at $p \leq 0.05$ for mean comparisons. Finally, ACC deaminase activity data were analyzed by Kruskal–Wallis test. The software INFOSTAT/Professional 1.1 [28] was used.

Results

Growth of *Azospirillum brasilense* strains on traditional and non-traditional media

The capability of the five *A. brasilense* to grow on traditional and non-traditional culture media was evaluated. The Sp7, Cd, 40M, and 42M strains showed typical colonies for *A. brasilense* in Nfb, RC, and TYG media. There were round, wrinkled dry and blue, scarlet red, and pink-colored colonies, in respective media (Supporting Information Fig. S2D, B, and C_1, respectively). In Nfb and RC media, the strain Az39 showed similar colony morphology as the other strains, but with a brighter halo at colony edges (Supporting Information Fig. S2A). Besides, Az39 strain showed white-colored colonies in TYG medium, instead of pink like as the others (Supporting Information Fig. S2C). In LGI ss, Nfb ss, and JNfb media, the five strains showed a veil-like pellicle with medium acidification or alkalinization, turning the dye from green to yellow or blue, respectively (Supporting Information Fig. S2G, E, and H, respectively). In LGglu ss and LGsuc ss media, the five strains did not show growth (Supporting Information Fig. S2F). The Sp7, Az39, 40M, and 42M strains showed little round, wrinkled dry and white-colored colonies in LGglu and LGsuc media (Supporting Information Fig. S2I and I_2). The Cd strain showed similar colony morphology but pink-colored instead of white (Supporting Information Fig. $S2I_1$). When agarose was used instead of agar-agar, the five strains showed the expected sub-superficial veil-like pellicle in Nfb ss-agarose and colonies development on Nfb-agarose with alkalinization of the media in both cases, indicating positive growth. Colonies morphology of Sp7, Az39, 40M, and 42M strains were the same as Nfb medium. Even after 15 days of incubation, the five strains absorbed BTB dye, showing blue-colored colonies. In LGglu-agarose medium, the five strains showed positive growth colonies with similar colony morphology as the colonies from LGglu medium. Finally, none of the strains showed positive growth on LG agarose medium.

Plant growth promotion mechanisms

Plant growth promotion mechanisms were also evaluated under *in vitro* conditions. The five strains showed siderophore production in CAS agar and did not show phosphate solubilization in Pikovskaya agar. The 42M strain produced higher levels of indolic compounds than 40M, Sp7, and Cd strains when the medium was supplemented with tryptophan as precursor (Table 1). Complementarily, when Nfb media were supplemented with ammonium chloride instead of tryptophan these strains produced in average 62% less amount of indolic compounds. Finally, in Nfb medium supplemented with ACC as sole N source, Sp7 strain showed higher ACC deaminase activity, estimated as absorbance values, than Az39 strain. Also, Cd, 40M, and 42M strains showed intermediate absorbance values (Table 1).

Community-level physiological profiles (CLPP)

The discriminant analysis of CLPP after 96 h of incubation showed that Az39 and Cd strains of *A. brasilense* exhibited differences in their physiology between each other and between the other three strains (Fig. 1). Axis 1 explained 72.8% of the total variance and was mainly composed of mannitol, proline, sorbitol, oxalic acid, glutamine, lactic acid, and arabinose. Axis 2 explained 20.6% of the total variance and was mainly composed of histidine and arabinose. The analysis of the number of CS used (Fig. 2) shows that the five strains used a high number of CS when incubation time increases. Between 48 and 72 h of incubation, the strains used a significantly

Table 1. Plant growth promotion potential activity of five *Azospirillum brasilense* strains.

	Strain				
	Sp7	Cd	Az39	40M	42M
Indolic compounds production ($\mu g\,10^{-6}\,UFC^{-1}$)	$0.50^{a} \pm 0.09$	$0.44^{a} \pm 0.01$	$0.60^{ab} \pm 0.01$	$0.50^{a} \pm 0.04$	$0.74^{b} \pm 0.07$
ACC deaminase activity	$3.90^{b} \pm 1.30$	$0.24^{ab} \pm 0.06$	$0.17^{a} \pm 0.17$	$0.37^{ab} \pm 0.03$	$0.22^{ab} \pm 0.01$

Indolic compounds production in flasks with NFb medium supplemented with tryptophan ($200\,\mu g\,ml^{-1}$). Mean values ± standard deviation. Different letters in superscript indicate significant differences in the average absorbance values between strains, with Tukey's test ($p \leq 0.05$). ACC deaminase activity estimated by growth obtained in wells with NFb medium with ACC as sole N source. Means of standardized absorbance values ± standard deviations. Different letters in superscript indicate significant differences in the average absorbance values between strains, with Kruskal–Wallis test ($p \leq 0.05$).

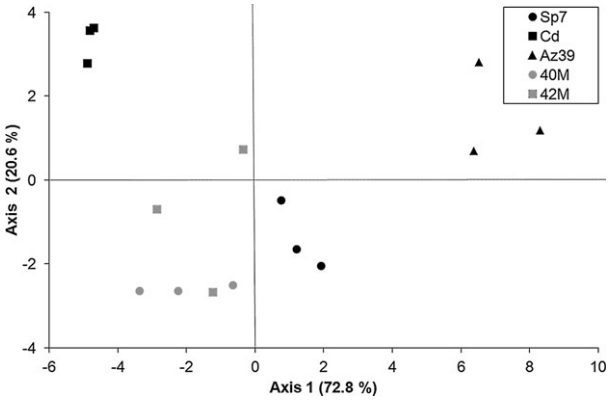

Figure 1. Discriminant analysis of CLPP of the five *Azospirillum brasilense* strains. The analysis was performed with absorbance values after 96 h of incubation. Each strain was cultivated in triplicates.

different number and kinds of CS but at 96 h of incubation, all the strains utilized among 9 and 12 of different CS (Fig. 2 and Table 2).

Bacterial cellular FAME analysis by GC

Table 3 shows that five fatty acids were present in all strains: octadecenoate (18:1), dodecanoate (12:0), hexadecenoate (16:1), hexadecanoate (16:0), and an unknown with 19 of equivalent chain length (ECL). Also present in some but not all the strains were: octadecanoate (18:0), 3-hydroxy-tetradecanoate (3-OH-14:0), tetradecanoate (14:0), and an unknown with 17 of ECL. Despite the fact that FAME profiles of all the strains analyzed was qualitatively similar, they could be grouped by differences in their composition performing a discriminant analysis of FAME data (Fig. 3). Axis 1 explained 58% of the total variance and was mainly composed of 12:0, 16:0, 16:1, and 18:1. Axis 2 explained 31% of the total variance and was mainly composed of 12:0 and ECL 19. Cd strain showed different FAME profile from the others (Fig. 3), with 18:1 between 53 and 66%, 16:1 plus 16:0 between 18 and 35%, minor quantities of 18:0, 12:0, 3-OH-14:0, 14:0, and the two unknowns of 17 and 19 of ECL (Table 3). Also, 42M strain showed different FAME profile from the others (Fig. 3), with 18:1 between 39 and 49%, lesser composition than the other strains, 16:1 plus 16:0 between 13 and 24%, 12:0 between 26 and 45%, and much higher composition than the other strains (Table 3). Sp7 and Az39 strains showed similar FAME profiles (Fig. 3) with 18:1 between 65 and 85%, 16:1 plus 16:0 between 4 and 11.5%, 12:0 between 7 and 16.5% (Table 3). Although 40M strain has a distinct FAME profile (Fig. 3), its composition showed intermediate percentage values in most of fatty acids (Table 3).

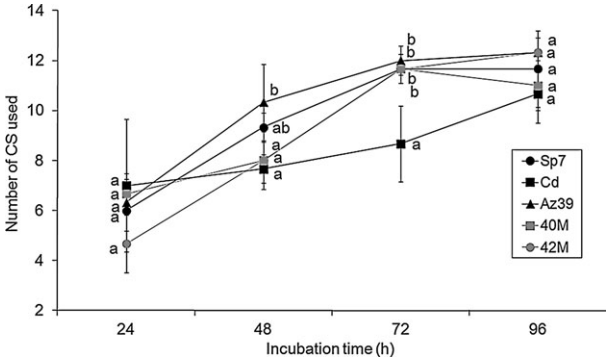

Figure 2. Number of carbon sources (CS) used by the five strains of *Azospirillum brasilense* analyzed. CS used means that the absorbance values were higher than AWCD of each strain at every reading time. Values are means ± standard deviations in triplicates. Different letters indicate significant differences between strains at each incubation time, with Tukey's test ($p \leq 0.05$).

Discussion

Historically, new strains of *A. brasilense* have been isolated and characterized on Nfb ss medium, by the typical growth as sub-superficial veil-like pellicle and medium alkalinization [3]. In this work, the five *A. brasilense* strains showed the typical veil-like pellicle and medium alkalinization in the Nfb ss. However, other authors could isolate diazotrophs different than *A. brasilense* using the Nfb ss medium [29, 30]. Generally, to confirm that the isolate is an *A. brasilense* strain, an aliquot of the typical pellicle in Nfb ss is inoculated on Nfb solid medium, supplemented with ammonium chloride (Nfb medium). *A. brasilense* has been reported as forming round, wrinkled and dry blue-colored colonies in this medium [3]. In Nfb medium, all the evaluated strains here showed typical colony morphology, but noteworthy the Az39 strain showed colony edge with a brighter halo.

The RC medium is used to facilitate the isolation of *Azospirillum* spp., because typical round, wrinkled, dry, and scarlet red-colored colonies were reported for *A. brasilense*. In this work, all the strains evaluated here showed typical colony morphology in RC medium, but noteworthy the Az39 strain showed colony edge with a brighter halo. This differential medium has the same composition than Nfb medium [1] but, instead of BTB, Congo red dye was used to increase selectivity for identifying the *Azospirillum* genus [5]. However, some authors used the RC medium for *Azospirillum* isolation and they found out typical scarlet red colonies which

Table 2. Absorbance values (590 nm) of the carbon sources (CS) used by *Azospirillum brasilense* strains at different time of incubation.

	CS	Sp7	Cd	Az39	40M	42M
48 h of incubation	Arabinose	$0.08^{a} \pm 0.01$	$0.22^{b} \pm 0.03$	$0.14^{ab} \pm 0.01$	–	$0.18^{b} \pm 0.03$
	Arginine	–	–	$0.16^{a} \pm 0.00$	$0.10^{a} \pm 0.00$	$0.09^{a} \pm 0.00$
	Citric acid	–	–	0.17 ± 0.01	–	–
	Fructose	$0.27^{a} \pm 0.03$	$0.23^{a} \pm 0.09$	$0.36^{a} \pm 0.05$	$0.24^{a} \pm 0.05$	$0.23^{a} \pm 0.02$
	Galactose	$0.08^{a} \pm 0.01$	–	$0.19^{b} \pm 0.01$	$0.17^{ab} \pm 0.05$	–
	Glycerin	$0.21^{a} \pm 0.01$	$0.34^{a} \pm 0.09$	$0.29^{a} \pm 0.05$	$0.22^{a} \pm 0.00$	$0.23^{a} \pm 0.02$
	Glutamine	$0.09^{a} \pm 0.00$	$0.21^{b} \pm 0.02$	$0.13^{a} \pm 0.02$	$0.11^{a} \pm 0.01$	$0.09^{a} \pm 0.00$
	Itaconic acid	–	–	0.13 ± 0.02	–	–
	Lactic acid	$0.26^{b} \pm 0.02$	$0.28^{b} \pm 0.04$	$0.17^{a} \pm 0.01$	$0.28^{b} \pm 0.01$	$0.28^{b} \pm 0.04$
	Malic acid	$0.08^{a} \pm 0.00$	$0.12^{a} \pm 0.03$	–	$0.09^{a} \pm 0.00$	$0.08^{a} \pm 0.00$
	Proline	$0.17^{ab} \pm 0.01$	$0.16^{a} \pm 0.09$	$0.35^{b} \pm 0.07$	$0.18^{ab} \pm 0.00$	$0.18^{ab} \pm 0.03$
	Xylose	0.08 ± 0.01	–	–	–	–
72 h of incubation	Arabinose	$0.08^{a} \pm 0.00$	$0.23^{b} \pm 0.04$	$0.14^{ab} \pm 0.03$	–	$0.15^{ab} \pm 0.02$
	Arginine	$0.08^{a} \pm 0.01$	–	$0.15^{b} \pm 0.00$	$0.10^{ab} \pm 0.01$	$0.09^{ab} \pm 0.01$
	Citric acid	$0.09^{a} \pm 0.01$	–	$0.14^{a} \pm 0.03$	-	–
	Fructose	$0.24^{b} \pm 0.01$	$0.36^{c} \pm 0.01$	$0.20^{ab} \pm 0.01$	$0.16^{a} \pm 0.03$	$0.18^{a} \pm 0.01$
	Galactose	$0.13^{a} \pm 0.05$	–	$0.16^{a} \pm 0.02$	$0.11^{a} \pm 0.01$	$0.08^{a} \pm 0.01$
	Glycerin	$0.18^{a} \pm 0.02$	$0.24^{a} \pm 0.05$	$0.22^{a} \pm 0.01$	$0.21^{a} \pm 0.00$	$0.21^{a} \pm 0.02$
	Glutamine	$0.07^{a} \pm 0.01$	$0.15^{b} \pm 0.01$	$0.11^{ab} \pm 0.01$	$0.09^{ab} \pm 0.01$	$0.08^{a} \pm 0.01$
	Itaconic acid	$0.11^{a} \pm 0.01$	$0.28^{c} \pm 0.01$	$0.20^{b} \pm 0.00$	$0.12^{a} \pm 0.03$	$0.09^{a} \pm 0.03$
	Lactic acid	$0.21^{a} \pm 0.02$	$0.16^{a} \pm 0.05$	$0.17^{a} \pm 0.01$	$0.16^{a} \pm 0.01$	$0.16^{a} \pm 0.02$
	Malic acid	$0.09^{a} \pm 0.02$	$0.10^{a} \pm 0.00$	$0.09^{a} \pm 0.00$	$0.08^{a} \pm 0.01$	$0.08^{a} \pm 0.01$
	Maltose	–	–	–	0.06 ± 0.01	–
	Proline	$0.16^{a} \pm 0.01$	–	$0.24^{b} \pm 0.01$	$0.19^{a} \pm 0.02$	$0.17^{a} \pm 0.01$
	Xylose	$0.13^{a} \pm 0.01$	$0.19^{a} \pm 0.09$	$0.17^{a} \pm 0.04$	$0.08^{a} \pm 0.01$	$0.09^{a} \pm 0.01$
96 h of incubation	Arabinose	$0.09^{a} \pm 0.02$	$0.18^{b} \pm 0.02$	$0.13^{ab} \pm 0.03$	–	$0.14^{ab} \pm 0.03$
	Arginine	$0.08^{a} \pm 0.01$	$0.14^{a} \pm 0.05$	$0.12^{a} \pm 0.02$	$0.90^{a} \pm 0.01$	$0.90^{a} \pm 0.02$
	Citric acid	–	$0.17^{b} \pm 0.05$	$0.12^{ab} \pm 0.03$	–	$0.07^{a} \pm 0.00$
	Fructose	$0.22^{bc} \pm 0.01$	$0.23^{c} \pm 0.02$	$0.20^{abc} \pm 0.01$	$0.15^{a} \pm 0.04$	$0.17^{ab} \pm 0.00$
	Galactose	$0.14^{a} \pm 0.04$	–	$0.16^{a} \pm 0.02$	$0.09^{a} \pm 0.01$	$0.12^{a} \pm 0.02$
	Glycerin	$0.17^{a} \pm 0.01$	$0.27^{a} \pm 0.08$	$0.20^{a} \pm 0.03$	$0.20^{a} \pm 0.01$	$0.19^{a} \pm 0.01$
	Glutamine	–	$0.11^{a} \pm 0.02$	$0.11^{a} \pm 0.04$	$0.08^{a} \pm 0.01$	$0.08^{a} \pm 0.01$
	Itaconic acid	$0.14^{a} \pm 0.03$	$0.28^{b} \pm 0.05$	$0.17^{a} \pm 0.01$	$0.16^{a} \pm 0.03$	$0.13^{a} \pm 0.02$
	Lactic acid	$0.22^{b} \pm 0.01$	$0.21^{b} \pm 0.02$	$0.18^{ab} \pm 0.01$	$0.14^{a} \pm 0.01$	$0.15^{a} \pm 0.03$
	Malic acid	–	–	$0.10^{a} \pm 0.01$	$0.07^{a} \pm 0.01$	$0.07^{a} \pm 0.00$
	Maltose	$0.08^{a} \pm 0.01$	$0.09^{a} \pm 0.02$	–	$0.07^{a} \pm 0.01$	–
	Proline	$0.14^{a} \pm 0.03$	–	$0.26^{b} \pm 0.01$	$0.17^{a} \pm 0.01$	$0.16^{a} \pm 0.01$
	Xylose	$0.13^{a} \pm 0.01$	–	$0.22^{b} \pm 0.01$	$0.11^{a} \pm 0.01$	$0.10^{a} \pm 0.02$

The (–) indicates that this CS had an absorbance value which was lower than the respective AWCD value. Means of absorbance values (at 590 nm) ± standard deviations. Standard deviations lower than 0.005 are shown as 0.00. Different letters in superscript indicate significant differences in the average absorbance values between strains for each CS, using the Tukey's test ($p \leq 0.05$).

Table 3. Percentage of cellular fatty acid composition of five strains of *Azospirillum brasilense*.

	Means of cellular fatty acid composition (%) ± standard deviations				
Fatty acids	Sp7	Cd	Az39	40M	42M
12:0	9.1 ± 2.1	1.0 ± 0.3	13.4 ± 3.1	13.8 ± 0.8	35.6 ± 9.1
14:0	nd	0.7 ± 0.4	nd	4.0 ± 1.0	nd
3-OH-14:0	nd	1.5 ± 0.3	nd	nd	nd
16:1	4.4 ± 0.6	14.5 ± 3.6	4.9 ± 0.9	6.6 ± 1.0	9.7 ± 3.2
16:0	0.5 ± 0.1	12.1 ± 4.8	0.7 ± 0.2	3.0 ± 0.9	8.7 ± 2.2
ECL 17	nd	1.2 ± 0.5	nd	nd	nd
18:1	81.7 ± 3.2	58.3 ± 5.7	76.8 ± 5.7	72.6 ± 7.1	44.1 ± 5.2
18:0	nd	1.8 ± 0.9	0.5 ± 0.1	2.1 ± 0.5	1.7 ± 0.1
ECL 19	1.8 ± 0.6	2.1 ± 0.9	3.8 ± 0.9	1.8 ± 0.7	1.2 ± 0.9

nd, not detected. Equivalent chain length (ECL) was used for unidentified fatty acids.

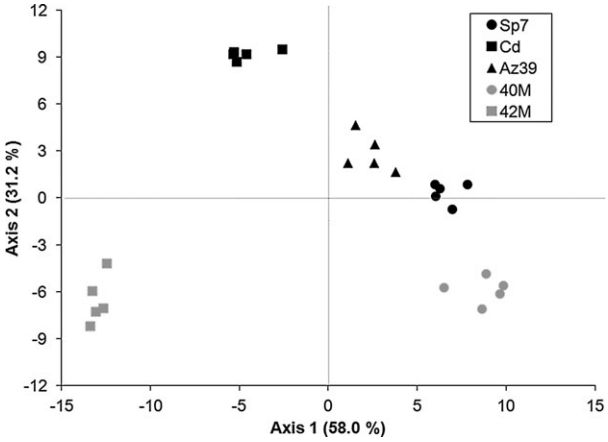

Figure 3. Discriminant analysis of FAME profiles of the five *Azospirillum brasilense* strains. The analysis was performed with percentages of fatty acid methyl esters composition. Each strain was analyzed in quintuplicates.

were identify as *Pseudomonas*, *Bacillus*, *Streptococcus*, and *Enterobacter* genera [29, 31, 32, 33].

The TYG medium has also been utilized by the authors as alternative to isolate representatives of the *Azospirillum* genus. It has been described that Cd is the only *A. brasilense* strain showing round, wrinkled, dry, and pink-colored colonies in this medium [23]. In this work, not only Cd strain but also Sp7, 40M and 42M strains showed typical colony morphology in this medium, while Az39 strain showed white-colored instead of pink-colored colonies.

We evaluated the capability of the five *A. brasilense* strains to grow into LGI ss and JNfb ss media, despite the fact that these media have been indicated for the isolation of *A. amazonense* and *Herbaspirillum* spp., respectively [3]. All the strains showed a sub-superficial veil-like pellicle in LGI ss and JNfb ss, with medium acidification or alkalinization, respectively. The composition of JNfb ss medium is similar to Nfb ss composition, but pH value is lower than the optimum range recommended for the isolation of *A. brasilense*. The LGI ss medium contains sucrose instead of glucose and its pH value is almost one unit bellow the recommended optimum pH for *A. brasilense*. However, differences in pH values of both media did not inhibit the growth of any *A. brasilense* strains. Besides, the five evaluated strains could grow and fix N_2 with sucrose as sole CS in LGI ss medium although it has been established that *A. brasilense* does not have these capabilities when sucrose is the sole available CS [3, 7, 10]. However, in LGglu ss and LGsuc ss media, none of the strains could grow probably due to the higher CS concentration in these media than those in LGI ss medium.

The five evaluated strains of *A. brasilense* were capable to grow on LGglu and LGsuc N-free solid media although it has been reported that *Azospirillum* genus does not have mechanisms to prevent inhibition of nitrogenase activity by O_2 [4]. In order to discard effects of any nutritional elements from agar-agar, some media were formulated with ultrapure agarose instead of agar-agar. We found that all the strains could grow on Nfb agarose and LGglu agarose media but they did not grow on N-free LG without any CS. These results indicates that agar-agar and agarose did not provide any C or N sources that could be used by the *A. brasilense* strains inoculated on LGglu and LGsuc media. In this regard, Aquilanti et al. [34] found that *A. brasilense*, *A. halopraeferans*, *A. amazonense*, and *A. lipoferum* formed colonies in N-free LG medium with sucrose as CS. Besides, some authors characterized the *A. brasilense* growth on N-free *Azotobacter* medium [35]. Some authors showed that carotenoids production contributed to the protection of nitrogenase enzyme against O_2 and keep it active [36]. However, other protective mechanisms which may cause differences in oxygen tolerance between strains are not well understood [37] and they remain to be studied.

All the *A. brasilense* strains produced siderophores in CAS medium. Other authors had reported siderophore production by several isolates of *A. brasilense* [25, 38]. Perrig et al. [39] have pointed out that Az39 and Cd strains did not have the capability to produce siderophores but they used different methodology to evaluate them. None of the evaluated strains showed phosphorous solubilization in Pikovskaya's agar although some strains of *A. brasilense* showed this capability [40, 41]. Same results were obtained for Az39 and Cd strains by Perrig et al. [39]. The five *A. brasilense* strains showed different levels of indolic compounds production when the medium was supplemented with tryptophane (Table 1) and lower amounts when they were incubated in tryptophane-free medium. Bashan et al. [20] showed that Sp7 and Cd strains produced this kind of substances. Other authors had reported that Sp7 strain could produce indolic compounds with [42, 43] and without [38, 44] tryptophane supplementation of the medium. It had pointed out that the indolic compounds production of some of the new *A. brasilense* strawberry isolates was lower than the level produced by Sp7 strain [38]. Besides, it was shown that the indolic compounds production of Cd strain was higher than the Az39 strain production [39] and the indolic compounds production of 40M strain was higher than Sp7 strain [42]. In this work, Cd and Az39 strains showed similar levels of indolic compounds production. Interestingly, 42M strain produced the highest level of this kind of substances, even than Sp7, Cd, and 40M strains.

Many authors have accepted that *Azospirillum* spp. did not produce ACC deaminase, based on Holguin and Glick [45] about *A. brasilense* Sp245 and Cd strains. However, some authors demonstrated ACC deaminase activity in *A. lipoferum* [46] and the *acdS* gene in *Azospirillum* species [47]. There is no report showing ACC activity of *A. brasilense* strains. This work shows for the first time that the five *A. brasilense* tested strains could have ACC deaminase activity (Table 1). Interestingly, Sp7 and Cd strains showed the highest and the lowest levels of ACC deaminase activity, respectively, estimated as absorbance values (Table 1). In this regard, it should be noted that these two strains are not very similar, contrary to what is generally accepted.

The CLPP have been traditionally used for the identification and classification of pure cultures and also have been used for the study of different microbial communities [27]. In this work, CLPP analysis with 28 different CS showed that Sp7, 40M, and 42M strains had similar physiological profiles (Fig. 1). Besides, Az39 and Cd strains showed different physiological profiles than the other three strains and between them (Fig. 1). Some authors showed certain genetic similarity between Sp7 and Cd strains [4, 15], but in this work we demonstrate physiological differences of both strains. Physiological differences between *Pseudomonas fluorescens* strains were reported before using the GN microplates and GN Biolog's computer software identification system (Biolog, Inc., Hayward, California, USA) [48]. However, this methodology was only applied to distinguish physiological differences of some *Azospirillum* species [49]. . Thus, this is the first report showing similarities and differences of the CLPP of a group of *A. brasilense* strains.

All the *A. brasilense* strains showed different number of CS used before the 96 h of incubation but they did not show differences after 96 h (Fig. 2). Despite this, all the strains used different CS and showed differences in the level of CS utilization (Table 2). Interestingly, the CLPP of particular strain could be explaining the capability of the strain to show a greater adaptation to variable environmental conditions. In the rhizosphere, the CLPP of the strain could define, among other processes, root colonization, biofilm formation, or biocontrol of plant diseases.

Some authors have observed that *Azospirillum* is capable of degrading petroleum present in contaminated soils and possible new species of *Azospirillum* have been isolated from petroleum contaminated soils [50]. Because of this, the possibility of incorporating xenobiotics as CS in microplates [27] allows the evaluation of the potential degrading capability of any bacterial species. In this work, the five *A. brasilense* strains showed lower absorbance values for glyphosate than its AWCD values, thus we concluded that *A. brasilense* strains did not utilize this CS. Despite this, Sp7 and Az39 strains showed higher absorbance values than 40M and 42M strains, at 48 h of incubation (data not shown). These results show that it could be interesting to perform more studies about the potential capability of *A. brasilense* strains to degrade xenobiotics.

As it was previously mentioned, *A. brasilense* does not grow when sucrose is the available CS. Although *A. brasilense* has the enzymes to metabolize glucose, this CS has a low rate of transport which impairs its cell uptake in liquid N-free media [51]. However, Goebel and Krieg [52] have pointed out that the growth of *A. brasilense* depends on the evaluated strain when the available CS is glucose. In this work, and unlike what it was found in solid and semisolid media, none of the strains grew in the wells of microplates with those two CS under our experimental condition (Table 2). Also, Tarrand et al. [4] and Hartmann and Zimmer [51] established that *A. brasilense* is not capable of utilizing mannitol and sorbitol. None of the strains were capable of using those CS available in microplates (Table 2). Some authors had also demonstrated that *Azospirillum* had the capability to fix N_2 using fructose, arabinose or galactose [9]. In this work, the five strains grew using fructose and galactose as CS, except Cd strain which did not grow with galactose as CS, and all of the strains, except 40M strain, grew with arabinose as CS (Table 2). Although all evaluated strains here correspond to *A. brasilense*, we observed that the use of some CS is not identical between them.

The five *A. brasilense* evaluated strains showed a similar pattern to that observed for several different strains of *A. brasilense* [14]. In general, the fatty acids pattern of this genus includes two major ones 18:1 *cis*-9 (52–63%) and 16:1 *cis*-9 (12–16.5%) and other seven fatty acids present in different amounts: 12:0, 14:0, 3-OH-14:0, 16:0, 18:0 and two unknown with 17 and 19 of ECL [14, 53]. Schenk and Werner [14] showed that the only two unsaturated fatty acids accounted for approximately 80% of the total fatty acid content and, although 18:0 is in minor percentage of total fatty acid content, it characterizes *A. brasilense*. However, these authors did not discuss the differences between strains for several *Azospirillum* species. Based on the differences observed in the composition of fatty acids of the strains studied here (Table 3) they could be classified in four different groups (Fig. 3). This work is the first report showing differences in composition of FAME between strains of *A. brasilense*. One example of these differences are related to the percentage of unsaturated fatty acids which were 85%

for Sp7 strain, 80% for both Az39 and 40M strains, 72% for Cd strain, and only 52% for 42M strain.

This work suggest that the five evaluated strains of *A. brasilense* have the capability of could be fixing N_2 under aerophilic conditions and can grow and could be fixing N_2 using CS previously considered inappropriate. Results obtained here reveal the need of further studies which could explain the metabolic pathways that enable *A. brasilense* to utilize some CS which could explain the growth of *A. brasilense* strains on N-free solid media in the presence of different O_2 gradients. Besides, this work showed the possibility to utilize the CLPP technique as a useful tool to detect physiological differences between strains of *A. brasilense* before the analyses with molecular techniques. This tool can be utilized also for the *in vitro* evaluation of potential degradation capability of xenobiotic compounds like glyphosate. Also, FAME profiles analysis can be used for strains differentiation. Interestingly, this work is the first report showing that *A. brasilense* could have has ACC deaminase activity.

The biochemical and physiological tests using cultivable methods are the main approach to isolate and characterize new strains. In this regard, the exhaustive knowledge revision and the differences between the five *A. brasilense* evaluated strains in this work, such as differences in CLPP, FAME profiles, and plant growth promotion mechanisms, have shown that it is not appropriate to make conclusions about bacterial species without analyzing several strains from different origins. Results obtained in this work revealed that it is necessary to continue developing studies and laboratory techniques focused on establishing a more appropriate protocol of isolation and characterization for new isolates under different environmental conditions. This will contribute to guarantee more efficient biotechnological processes, inoculation success, and agriculture sustainability.

Acknowledgments

Authors want to thank the collaboration of Dr. Raúl Pedraza (UNT, Tucumán, Argentina) to provide the CAS plates, Lic. Florencia D'Auria for her suggestions and Ing. Agr. Alejandro Perticari (IMyZA, INTA, Argentina) for providing the Az39 strain. We are especially grateful to Dr. Yoav Bashan for his suggestions and comments about this manuscript. This work was partially supported by the grants: FONCYT 2008 PICT1864 from the MINCyT, UBACyT project 20020090100255, Universidad de Buenos Aires, Argentina and CYTED/CNPq (490013/2010-4). We are also are grateful to editors and anonymous reviewers for their comments and suggestions.

References

[1] Bashan, Y., Trejo, A., de-Bashan, L.E., 2011. Development of two culture media for mass cultivation of *Azospirillum* spp. and for production of inoculants to enhance plant growth. Biol. Fertil. Soils, **47**, 963–969.

[2] García de Salamone, I.E., Di Salvo, L.P., Escobar Ortega, J.S., Boa Sorte, P.M.F. et al. 2010. Field response of rice paddy crop to *Azospirillum* inoculation: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants. Plant Soil, **336**, 351–362.

[3] 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, London, 134–141.

[4] Tarrand, J.J., Krieg, N.R., Döbereiner, J., 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol., **24**, 967–980.

[5] Rodríguez-Cáceres, E.A., 1982. Improved medium for isolation of *Azospirillum* spp. Appl. Environ. Microbiol., **44**, 990–991.

[6] Puente, M.L., García, J.E., Perticari, A., 2008. Investigación aplicada de *Azospirillum* para su uso como promotor del crecimiento en cultivos de interés agronómico, in: Cassán, F.C., García de Salamone, I.E. (Eds.), *Azospirillum* sp.: Cell Physiology, Plant Interactions and Agronomic Research in Argentina. Asociación Argentina de Microbiología, Buenos Aires, Argentina, 167–178.

[7] Bergey, D.H., Holt, J.G., 1994. Bergey's Manual of Determinative Bacteriology, 9th edn., Williams and Wilkins, Baltimore, MD, USA.

[8] Zhang, Y., Burris, R.H., Ludden, P.W., Roberts, G.P., 1997. Regulation of nitrogen fixation in *Azospirillum brasilense*. FEMS Microbiol. Lett., **152**, 195–204.

[9] Martínez-Drets, G., Del Gallo, M., Burpee, C., Burris, R.H., 1984. Catabolism of carbohydrates and organic acids and expression of nitrogenase by Azospirilla. J. Bacteriol., **159**, 80–85.

[10] Gillis, M., Reinhold-Hurek, B., 1994. Taxonomy of *Azospirillum*, in: Okon, Y. (Ed.), *Azospirillum*/Plant Associations. CRC Press, USA, 1–14.

[11] Maas, J.L., Finney, E.L., Civerolo, E.L., Sasser, M., 1985. Association of an unusual strain of *Xanthomonas campestris* with apple. Phytopathol., **75**, 439–445.

[12] Jarvis, B.D.W., Tighe, S.W., 1994. Rapid identification of *Rhizobium* species based on cellular fatty acid analysis. Plant Soil, **161**, 31–41.

[13] Graham, P.H., Sadowsky, M.J., Tighe, S.W., Thompson, J.A. et al., 1995. Differences among strains of *Bradyrhizobium* in fatty acid-methyl ester analysis. Can. J. Microbiol., **41**, 1038–1042.

[14] Schenk, S.U., Werner, D., 1988. Fatty acids analysis of four *Azospirillum* species reveals three groups. Arch. Microbiol., **149**, 580–582.

[15] Gündisch, C., Kirchhof, G., Baur, M., Bode, W. et al., 1993. Identification of *Azospirillum* species by RFLP and pulsed-field gel electophoresis. Microbiol. Releases, **2**, 41–45.

[16] Kirchhof, G., Schloter, B., Aßmus, B., Hartmann, A., 1997. Molecular microbial ecology approaches applied to diazotrophs associated with non-legumes. Soil Biol. Biochem., **29**, 853–862.

[17] Baudoin, E., Couillerot, O., Spaepen, S., Moënne-Loccoz, Y. et al., 2010. Applicability of the 16S-23S rDNA internal spacer for PCR detection of the phytostimulatory PGPR inoculants *Azospirillum lipoferum* CRT1 in field soil. J. Appl. Microbiol., **108**, 25–38.

[18] Lin, S.Y., Shan, F.T., Young, C.C., 2011. Rapid detection and identification of the free-living nitrogen fixing genus *Azospirillum* by 16S rRNA-gene-targeted genus-specific primers. Antoine Van Leeuwenhoek J. Microbiol., **99**, 837–844.

[19] Di Salvo, L.P., Escobar Ortega, J.S., García de Salamone, I.E., 2009. Isolation of microaerophilic N2-fixing bacteria from rice cultivated in Entre Ríos, Argentina. 8th International PGPR Workshop. Portland, Oregon, USA. http://www.umc.edu.dz/vf/images/abstract%20booklet1.pdf

[20] Bashan, Y., Holguin, G., de-Bashan, L.E., 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can. J. Microbiol., **50**, 521–577.

[21] Naiman, A.D., Latrónico, A., García de Salamone, I.E., 2009. Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: impact on the production and culturable rhizosphere microflora. Eur. J. Soil Biol., **45**, 44–52.

[22] Tamura, K., Peterson D., Peterson N., Stecher G. et al., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., **28**, 2731–2739.

[23] Leyva, L.A., Bashan, Y., 2008. Activity of two catabolic enzymes of the phosphogluconate pathway in mesquite roots inoculated with *Azospirillum brasilense* Cd. Plant Physiol. Biochem., **46**, 898–904.

[24] Gaur, A.C., 1990. Physiological functions of phosphate solubilizing micro-organisms, in: Gaur, A.C. (Ed.), Phosphate Solubilizing Microorganisms as Biofertilizers. Omega Scientific Publishers, New Delhi, India, 16–72.

[25] Tortora, M.L., Díaz-Ricci, J.C., Pedraza, R.O., 2011. *Azospirillum brasilense* siderophores with antifungal activity against *Colletotrichum acutatum*. Arch. Microbiol., **193**, 275–286.

[26] Hynes, R.K., Leung, G.C.Y., Hirkala, D.L.M., Nelson, L.M., 2008. Isolation, selection, and characterization of beneficial rhizobacteria from pea, lentil, and chickpea grown in western Canada. Can. J. Microbiol., **54**, 248–258.

[27] Di Salvo, L.P., García de Salamone, I.E., 2012. Evaluation of soil-microbial communities by their CLPP. Standardization of a laboratory technique to replace commercial available microplates. Aust. Ecol., **22**, 129–136.

[28] Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L. et al., 2011. InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

[29] Fischer, S.E., Fischer, S.I., Magris, S., Mori, G.B., 2007. Isolation and characterization of bacteria from the rhizosphere of wheat. World J. Microbiol. Biotechnol., **23**, 895–903.

[30] Tripathi, A.K., Verma, S.C., Ron, E.Z., 2002. Molecular characterization of a salt-tolerant bacterial community in the rice rhizosphere. Res. Microbiol., **153**, 579–584.

[31] Ogbo, F., Okonkwo, J., 2012. Some characteristics of a plant growth promoting *Enterobacter* sp. isolated from the roots of maize. Adv. Microbiol., **2**, 368–374.

[32] Pisarska, K., Adamski, M., Pietr, S.J., 2011. Lack of nifH gene in some endophytic bacteria isolated on RC solid medium. Commun. Agric. Appl. Biol. Sci., **76**, 699–704.

[33] Tiwari, P., Tripathi, J., Ekka, S.R., 2011. *Azospirillum* spp. isolated from Raigarh District of Chhattisgarh. J. Phytol., **3**, 24–26.

[34] Aquilanti, L., Favilli, F., Clementi, F., 2004. Comparison of different strategies of isolation and preliminary identification of *Azotobacter* form soil samples. Soil Biol. Biochem., **36**, 1475–1483.

[35] Salantur, A., Ozturk, A., Akten, S., 2006. Growth and yield response of spring wheat (*Triticum aestivum* L.) to inoculation with rhizobacteria. Plant Soil Environ., **52**, 111–118.

[36] Marchal, K., Vanderleyden, J., 2000. The "oxygen paradox" of dinitrogen-fixing bacteria. Biol. Fertil. Soils, **30**, 363–373.

[37] Hartmann, A., 1988. Ecophysiological aspects of growth and nitrogen fixation in *Azospirillum* spp. Plant Soil, **110**, 225–238.

[38] Pedraza, R.O., Motok, J., Tortora, M.L., Salazar, S.M. et al., 2007. Natural occurrence of *Azospirillum brasilense* in strawberry plants. Plant Soil, **295**, 169–178.

[39] Perrig, D., Boiero, M.L., Masciarelli, O.A., Penna, C. et al., 2007. Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. Appl. Microbiol. Biotechnol., **75**, 1143–1150.

[40] Puente, M.E., Bashan, Y., Li, C.Y., Lebsky, V.K., 2004. Microbial populations and activities in the rhizoplane of rock-weathering desert plants I. Root colonization and weathering of igneous rocks. Plant Biol., **6**, 629–642.

[41] Rodriguez, H., Gonzalez, T., Goire, I., Bashan, Y., 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften, **91**, 552–555.

[42] Baca, B.E., Jijón-Moreno, S., Pedraza, R.O., Campero-Romero, A.N. et al., 2012. Genetic diversity of genes involved in indole-3-acetic acid production in the plant growth promoting bacterium *Azospirillum*. 13th Symposium on Biological Nitrogen Fixation with Non-Legumes. Neuherberg, Munich, Germany.

[43] Pedraza, R.O., Ramirez-Mata, A., Xiqui, M.L., Baca, B.E., 2004. Aromatic amino acid aminotransferase activity and indole-3-acetic acid production by associative nitrogen-fixing bacteria. FEMS Microbiol. Lett., **233**, 15–21.

[44] Dobbelaere, S., Croonenborghs, A., Thys, A., Vande Broek, A. et al., 1999. Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil, **212**, 155–164.

[45] Holguin, G., Glick, B.R., 2001. Expression of the ACC deaminase gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. Microb. Ecol., **41**, 281–288.

[46] Esquivel-Cote, R., Ramírez-Gama, R.M., Tsuzuki-Reyes, G., Orozco-Segovia, A. et al., 2010. *Azospirillum lipoferum* strain AZm5 containing 1-aminocyclopropane-1-carboxylic acid

deaminase improves early growth of tomato seedlings under nitrogen deficiency. Plant Soil, **337**, 65–75.

[47] Blaha, D., Prigent-Combaret, C., Sajjad, M.M., Moënne-Loccoz, Y. 2006. Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene *acdS* in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiol. Ecol., **56**, 455–470.

[48] García de Salamone, I.E., Hynes, R.K., Nelson, L.M., 2001. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Can. J. Microbiol., **47**, 404–411.

[49] Eid, M.M.A., Sherwood, J.E., 1995. Pulsed-field gel electrophoresis fingerprinting for identification *of Azospirillum* species. Curr. Microbiol., **30**, 127–131.

[50] Reis, V.M., dos Santos Teixeira, K.R., Pedraza, R.O., 2011. What is expected from the genus *Azospirillum* as a plant growth-promoting bacteria? in: Mahashwari, D.K. (Ed.), Bacteria in Agrobiology: Plant Growth Responses. Springer-Verlag, Berlin, 123–138.

[51] Hartmann, A., Zimmer, W., 1994. Physiology of *Azospirillum*, in: Okon, Y. (Ed.), *Azospirillum*/Plant Associations. CRC Press, USA, 15–40.

[52] Goebel, E.M., Krieg, N.R., 1984. Fructose catabolism in *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol., **159**, 86–92.

[53] Olubayi, O., Caudales, R., Atkinson, A., Neyra, C., 1998. Differences in chemical composition between nonflocculated and flocculated *Azospirillum brasilense* Cd. Can. J. Microbiol., **44**, 386–390.