

Artículo científico

A survey of denitrifying *Azospirillum brasilense* in two contrasted conditions of the sugarcane cropping region of Tucumán, Argentina

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Abstract

The aim of this work was the assessment of denitrifying *Azospirillum brasilense* isolates in two contrasted regions in the sugarcane cropping region of Tucumán, Argentina. For that, *Azospirillum* was isolated from the rhizoplane of sugarcane plants using nitrogen-free malate semisolid medium. Microbiological characteristics and restriction profiles of 16S rDNA indicated the presence of *A. brasilense*. PCR analysis of the isolates showed that the key enzyme for denitrification corresponded to the nitrite reductase type *nirS*. There were differences among the isolates in their physiological response to the nitrite reductase activity and the enzymatic restriction profiles of the *nirS* fragment. Differences among the isolates at genomic level were also assessed by RAPD method. According to the higher naturally occurrence of denitrifying *Azospirillum* in the wetter area of the sugarcane cropping region, our results suggest the existence of an influence of rainfall regime and soil characteristics in their environmental distribution. The knowledge of the ecology of denitrifiers (in this case, *Azospirillum* strains), represents a step forward to the rational utilization of microbial diversity in agriculture.

Keywords: *Azospirillum brasilense*, denitrification, *nirS*, nitrite reductase, sugarcane.

Resumen

El objetivo de este trabajo fue evaluar aislamientos desnitrificadores de *Azospirillum* en dos regiones cañeras contrastantes de Tucumán, Argentina. Para ello, *Azospirillum* fue aislado del rizoplasma de caña de azúcar en medio de cultivo semisólido, libre de nitrógeno. La caracterización microbiológica y los perfiles de restricción de gen 16S ADNr indicaron la presencia de *A. brasilense*. El análisis por PCR mostró que la enzima clave para la desnitrificación corresponde a la nitrato reductasa tipo *nirS*. Los aislamientos mostraron distinta respuesta fisiológica en la actividad nitrato reductasa y en los perfiles de restricción enzimática de *nirS*. La diferencia entre los aislamientos, a nivel genómico, también fue evaluada por RAPD. De acuerdo con la mayor incidencia natural desnitrificadora de *Azospirillum* en la zona más húmeda (NMP) de la región cañera, estos resultados sugieren la existencia de una influencia del régimen de lluvias y de las características del suelo en su distribución ambiental. El estudio de la ecología de los desnitrificadores (en este caso, cepas de *Azospirillum*), contribuye a ampliar el conocimiento sobre la diversidad microbiana en la agricultura.

Palabras clave: *Azospirillum brasilense*, desnitrificación, *nirS*, nitrato reductasa, caña de azúcar.

Introduction

The genus *Azospirillum* belongs to the group of plant growth promoting bacteria (PGPB), capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance through different mechanisms (Bashan and de-Bashan, 2010).

Several authors reported significant contribution of biological nitrogen fixation associated with roots of sugarcane (Lima *et al.*, 1987; Boddey *et al.*, 1991; Urquiaga *et al.*, 1992; Boddey *et al.*, 1995); however, some associative

bacteria can decrease the potential plant growth by denitrification. (Zimmer *et al.*, 1995). In this process the nitrate is reduced via nitrite to gaseous nitrogenous compounds (NO, N₂O and N₂), which are lost as N source for plants (Zimmer *et al.*, 1995). Denitrification is a common feature among many bacteria (Knowles, 1982) and was reported to be carried out by different species and strains of *Azospirillum* (Neyra *et al.*, 1977; Tarrand *et al.*, 1978; Döbereiner 1992; Zimmer *et al.*, 1995; Kloos *et al.*, 2001). Considering that this process causes major nitrogen losses in agricultural soils to which fertilizers are applied, the

non-denitrifying strains of the root associated bacterium *Azospirillum* are believed to be more beneficial for plant growth promotion (Zimmer *et al.*, 1995).

Nitrite reductase is the key enzyme in the dissimilatory denitrification process. The reduction of nitrite to NO can be catalyzed by the products of two different nitrite reductase genes: the *nirK* product, that contains copper, and the *nirS* product, that contains cytochrome cd_1 (Coyne *et al.*, 1989). Although structurally different, both enzyme types are functionally and physiologically equivalent (Glockner *et al.*, 1993; Zumft, 1997). It was reported that *A. brasilense*, *A. lipoferum* and *A. halopraeferens* possess a cytochrome cd_1 containing enzyme catalyzing the reduction of nitrite to NO from the *nirS* gene (Bothe *et al.*, 1994; Braker *et al.* 1998; Braker *et al.* 2000; Kloos *et al.*, 2001), while *A. irakense* and *A. doebereineriae* have a Cu-containing nitrite reductase and *A. amazonense* is unable to denitrify (Kloos *et al.*, 2001).

Changes in denitrifying activity may be caused by cycles of wetting and drying, which promote nitrification followed by denitrification (Reddy and Patrick, 1975). Differences in microbial community enzyme activity influence the response of N_2O mole fraction to rain events, contributing to explain denitrification differences (Bergsma *et al.*, 2002).

In this work we investigated the occurrence of denitrifying *Azospirillum* isolates in two contrasting environmental situations within the same sugarcane-cropping region. Differences among isolates were evaluated by analyzing the nitrite reductase activity assay, PCR amplification of the *nirS* and *nirK* genes, followed by enzyme digestion, as well as by using the random amplified polymorphic DNA (RAPD) methodology.

Materials and methods

Area of sampling

The sugarcane cropping region in the province of Tucumán, (NorthWest of Argentina), is located between 26° 31' and 27° 40' South latitude, a subtropical region with monzonic climate and a mean annual temperature of 26°C. The altitude above sea level increases from East to West (350 m to 750 m, respectively) within this region (Torres Bruchmann, 1979). Based on hydrological and soil characteristics the sugarcane-cropping region was divided into two areas, West and East, from where *Azospirillum* was isolated. Data of the mean annual rainfall and evapotranspiration in Tucumán were obtained from long-term re-

cords from the Agro-meteorology Section of the Estación Experimental Agroindustrial Obispo Colombres (EEAOC). Values of the predominant soil type, texture, soil pH (1:2.5 in H_2O), mean organic matter content and carbon-nitrogen ratio were taken from reported data for the soils of the sugarcane region in this province (Zuccardi and Fadda, 1972), and from the Soil Section of the EEAOC (Table 1).

Strain isolation and identification

In order to compare two contrasted areas, we considered *Azospirillum* isolated from the rhizoplane of sugarcane roots, as they are more exposed to environmental conditions than endophytic strains, which may be buffered by apoplastic fluids inside the roots.

Fourteen locations were sampled: seven in each area (West and East). They were selected by counterbalancing their geographical distribution along the main isohietes across the sugarcane-cropping region, which involve the two contrasting areas.

Roots of sugarcane plants were collected during summer season. Samples consisted of six subsamples (10 g of roots) collected at random, which were mixed together before assay. They were kept at 4° C in sterile plastic bags until they were processed within the 24 h of obtained. *Azospirillum* was isolated from washed roots of sugarcane, according to Döbereiner *et al.* (1995). Root segments were cut into 1 cm long pieces with a sterile scissors, aseptically transferred to N-free malate semisolid medium (NFb), (Döbereiner *et al.*, 1995) and incubated 36-72 h at 30°C.

Cultures forming a typical white pellicle below the surface of the medium were streaked out on Petri dishes containing NFb solid medium supplemented with yeast extract (0.5 g l⁻¹). Individual colonies were newly transferred into vessels with NFb semisolid medium and incubated at 30°C for 72 h. After purification, the isolates were subjected to identification. 140 isolates (10 from each of the fourteen localities sampled) were considered in this study.

The identification of the isolates was based on colony morphology in culture media, biochemical tests reported for *Azospirillum* species (Tarrand *et al.*, 1978; Döbereiner *et al.*, 1995), and a molecular approach (Grifoni *et al.*, 1995). The strains *A. brasilense* Sp7 (ATCC 29145), *A. lipoferum* Sp59 (ATCC 29707), *A. amazonense* Y1 (ATCC 35119), *A. irakense*, and *A. halopraeferens*, used as references in the identification of the local isolates, were kindly provided by EM-BRAPA-Agrobiología, Rio de Janeiro, Brazil.

Table 1. Characteristics of the two sampling areas considered for the isolation of *Azospirillum* in the sugarcane cropping region of Tucumán, Argentina.

Characteristic	West area	East area
Mean annual rainfall	1339.19 mm	797.18 mm
Mean annual evapotranspiration (potential)	900 mm (or less)	1046 mm
Hydric balance	439.19 mm	-248.82 mm
Predominant soil type	Typic Argiudoll – Fluventic Hapludoll	Typic ustifluent – Thapto-Argic Hapludoll
Predominant soil texture	Loamy	Clay loam – Silty loam
Soil pH	6.0 – 7.0	6.0 – 7.5
Mean organic matter content	2 – 6%	3 – 5%
C/N	10 – 12	10 – 12

Molecular identification

To identify the local isolates of *Azospirillum* a fragment of the gene 16SrDNA was analyzed. DNA used as template was obtained by thermal disruption of cells of different isolates. Cell suspension (6 µl) coming from NFb semisolid medium were thoroughly suspended in 30 µl bidistilled sterile water, boiled at 95°C for 10 minutes, and cooled down at room temperature. 1 µl of this suspension was used in the PCR reactions.

A fragment of the 16S rDNA gene from each isolate was amplified with specific primers (27f and 1495r), according to the protocol of Grifoni *et al.* (1995). The amplification products were digested with 5 U µl⁻¹ of the endonuclease *AluI* (Promega). Two PCR reactions were performed from the same sample. Negative controls, without DNA, were included in each experiment. The digestion products were analyzed by horizontal agarose (1.5%) gel electrophoresis (4 V cm⁻¹) in TBE 1X (Tris-borate-EDTA) running buffer (Sambrook *et al.*, 1989). Gels were stained with ethidium bromide (0.5 µg ml⁻¹) for 30 min, washed three times with distilled water and visualized on UV analyzer. Digital images were acquired by using the 1D Image Analysis Software (Kodak Digital Science™) with digital camera.

Nitrite reductase activity

All the isolates were assessed for nitrite reductase activity (nir). The test was performed as indicated by Döbereiner (1980). Each isolate was assessed by inoculating 50 µl of actively growing cells in N-free malate semisolid media (NFb) into new semisolid NFb medium containing 5 mM NH₄NO₃. Cultures were incubated for 24-48 h at 30°C. When the typical pellicle developed by *Azospirillum* in NFb has just reached the top of the medium, the culture was mixed thoroughly to break the pellicle and incubated at 30°C. The presence of bubbles in the culture medium after 1 h from the pellicle disruption in five replicates was

considered a positive reaction for denitrification. *A. brasilense* Sp7 was used as positive control for denitrification.

The nir test was used also to calculate the most probable number (MPN) of denitrifying *Azospirillum* from root samples of each locality of the two regions considered in this work, following the prescriptions of Döbereiner *et al.* (1995) and by using the McCrady table for 5 replicates.

PCR amplification of a nirS/nirK fragments and enzyme digestion

DNA used as template was obtained by thermal disruption of cells of different isolates as previously explained. 1 µl of this suspension was used in the PCR reactions. The amplification conditions and the primers used were according to Braker *et al.* (1998). PCR was repeated three times; the strain Sp7 of *A. brasilense* was used as positive control and the negative was the mix without DNA.

From the amplification product, 7 µl were digested with the endonucleases *AccI*, *SalI* and *SmaI* (5 U µl⁻¹), (Promega). These enzymes were chosen using the Webcutter software (<http://www.firstmarket.com/cgi-bin/firstmarket/cutter>). The digestion products were analyzed by horizontal agarose (1.5%) gel electrophoresis (4 V cm⁻¹) in TBE 1X running buffer (Sambrook *et al.*, 1989). Gels were stained, washed and digitalized as previously explained.

Random amplified polymorphism of DNA (RAPD)

For the analysis of the genomic variability among isolates, RAPD was used (Williams *et al.* 1990). Microbial samples for DNA extraction were obtained from suspensions of cells grown in liquid NFb medium supplemented with 1 g l⁻¹ NH₄Cl. Bacterial DNA was obtained as described by Sambrook *et al.* (1989) and purified with the Wizard™ Genomic DNA Purification Kit (Pro mega). The DNA was quantified with a Beckman 7000 spectrophotometer and polymers-

rase chain reactions were carried out with 20 ng of DNA samples. Reaction mixtures were prepared in 20 μ l containing: 0.5 mM MgCl₂, 0.1 mM of each dNTP (deoxyribonucleotides), 0.2 μ M of random sequence 10-mer primers: A06 GAG-TCTCAGG (Biodynamics), A08 ACGCACA-ACC (Biodynamics), OPJ12 GTCCCGTGGT (Operon Technologies), OPJ19 GGACACCACT (Operon Technologies), OPJ20 AAGCGGCCTC (Operon Technologies), 0.75 Units of Taq polymerase (Promega) and 2 μ l of Taq buffer (10X). The program used was: denaturation, 30 sec at 92°C; annealing, 1 min at 35°C; extension, 2 min at 72°C; number of cycles: 45. The program started with a thermal treatment of 3 min at 94°C and finished with an extension of 5 min at 72°C. The reaction was carried out in a MJ Research Thermocycler. Amplification products were separated by electrophoresis at 4 V cm⁻¹ in a 1.5 % agarose gel in TBE 0.5X running buffer. Gels were stained, washed and digitalized as previously explained. RAPD experiments were repeated three times to avoid false results and to ensure reproducibility. Negative controls (mix without DNA), were included in each experiment.

Results

Bacterial growth in NFb semisolid medium was observed in all samples collected from sugarcane roots. They showed Gram-negative staining, motility and S-shape cells observed by phase-contrast microscopy. Colonies formed on NFb solid medium supplemented with yeast extract showed dry consistency, with round form and wrinkled edges. According to these characteristics, including biochemical test, and comparison with the positive controls we identified the species *A. brasilense* (data not shown). These results were confirmed by restriction profiles of a 1450 bp fragment of the gene 16SrDNA amplified by PCR and digested with *AluI*, using the strain *A. brasilense* Sp7 as positive control. Figure 1 shows the restriction profiles of some of the local isolates with the endonuclease *AluI* in comparison with the reference strain *A. brasilense* Sp7.

PCR amplifications of *nirS* and *nirK* were carried out with all the isolates, regardless if they were denitrifiers or not (*nir*⁺ or *nir*⁻). Only those isolates that were *nir*⁺ in the biochemical test harbored a fragment of the *nirS* gene (890 bp) (Figure 2), in agreement with previous reports (Braker *et al.*, 1998; Braker *et al.*, 2000). No amplification signal was observed for *nirK*.

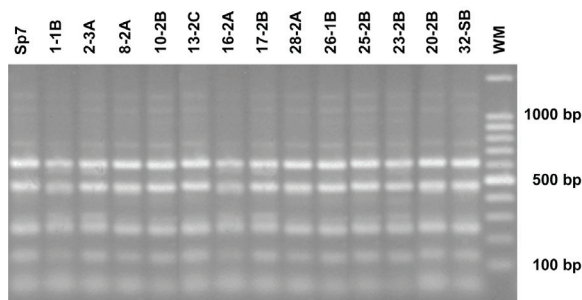


Fig. 1. Electrophoresis of amplified 16S rDNA fragments digested with endonuclease *AluI*. Profiles correspond to local *A. brasilense* isolates from the sugarcane cropping region of Tucumán, Argentina. Sp7 is the strain of reference for *A. brasilense*. WM: molecular weight marker Ladder 100 bp (Promega).

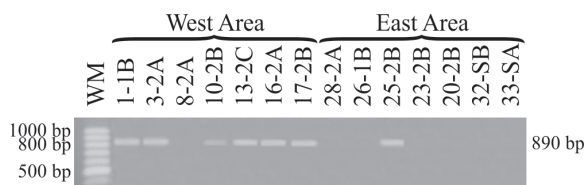


Fig. 2. Electrophoresis of amplified *nirS* fragment assessed on fourteen *Azospirillum brasilense* local isolates of the sugarcane area of Tucumán, Argentina. WM: Molecular weight marker Ladder 100 bp.

In Table 2 we present the MPN of denitrifiers found in the West and East areas of the sugarcane cropping region of Tucumán, Argentina, as well as the results of nitrite reductase activity assay carried out with the isolates of the fourteen localities sampled. Although the presence of bubbles in five replicates was considered a positive reaction for denitrification, the amount and size of them were not the same, varying among the isolates.

Table 2. MPN of denitrifiers and nitrite reductase activity test (*nir*) of *Azospirillum* isolated from fourteen localities within two areas in the sugarcane region of Tucumán, Argentina.

West area			East area		
Locality sampled	MPN denitrifiers	<i>nir</i> activity*	Locality sampled	MPN denitrifiers	<i>nir</i> activity*
Las Talitas	8x10 ⁴	+	Los Ralos	n.d.	-
Lules	7x10 ⁴	+	Ranchillos	n.d.	-
Berdina	6x10 ⁵	+	Agua Dulce	6x10 ²	+
Maldonado	7x10 ⁵	+	Tres Pozos	n.d.	-
Arcadia	8x10 ⁵	+	Leales	n.d.	-
Los Sarmientos	6x10 ⁵	+	Atahona	n.d.	-
Alberdi	6x10 ⁴	+	Monteagudo	n.d.	-
Total			Total		

*: Performed with ten *Azospirillum* isolates from each locality.

n.d.: not detected.

+: positive nitrite reductase activity, detected by the production of bubbles in NFb semisolid media.

-: negative nitrite reductase activity, evaluated as the absence of bubbles in NFb semisolid media.

Figure 3 shows the restriction profiles of the *nirS* fragment of some isolates, amplified by PCR and digested with *AccI*, *SalI* and *SmaI* (Promega).

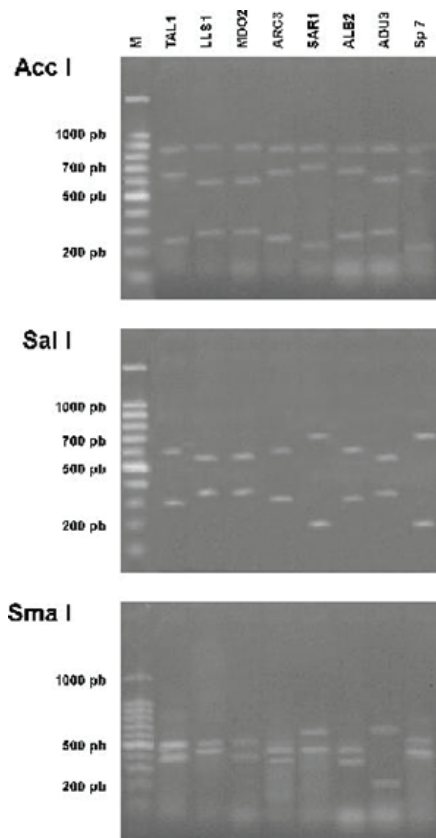


Fig. 3. Electrophoresis of amplified *nirS* fragments digested with *AccI*, *SalI* and *SmaI*. 1-1B, 3-2A, 10-2B, 13-2C, 16-2A, 17-2B and 25-2B are designations of *A. brasilense* isolates from the sugarcane area in Tucumán, Argentina. Sp7 is the strain of reference for *A. brasilense*. WM: Molecular weight marker Ladder 100 bp.

Whereas variability in the enzyme digestion profile of the *nirS* fragment was observed, the RAPD technique revealed an intra-specific genomic variability (Fig. 4).

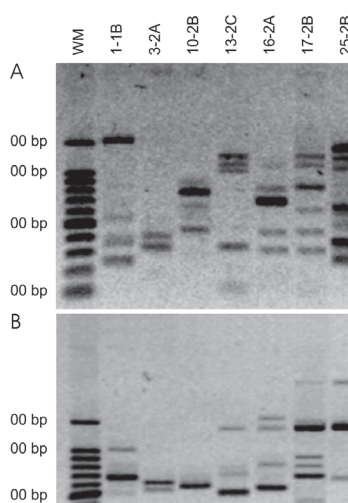


Fig. 4. RAPD profiles of the amplification of genomic DNA of seven *A. brasilense* local isolates. All showed positive nitrite reductase activity (*nir*⁺) and exhibited PCR-*nirS* amplification. A and B correspond to the amplification reaction using the primers A-06 and A-08 respectively.

Discussion

In this work we isolated *Azospirillum* from the rhizoplane of sugarcane plants in two contrasted sugarcane cropping areas of Tucumán, Argentina, and identified the species *A. brasilense*. According to the MPN of denitrifiers, the higher values were found in the wetter area (West) under study, compared with the drier one (East). Considering that only a small percentage of bacteria can be cultivated as yet, the MPN for denitrifiers may represent only a small percentage of bacteria, including the overall denitrifiers. Comparing the two regions of sampling, *A. brasilense* isolates with denitrifying activity was predominant in the West area. Since denitrifying bacteria consume easily available organic matter while using nitrates as their electron acceptor, we speculate that the organic matter content and carbon-nitrogen ratio (Table 1) would not be limiting factors for denitrification. Table 1 shows that these parameters are almost similar in both areas, as well as pH values.

When flooding occurs, as it happens in many parts of the western sugarcane area of Tucumán during summer season, the water covers the soil and hinders the oxygen supply; consequently, bacteria start utilizing nitrogenous compounds as electron acceptors contributing to the denitrification of soils. Thereby, available nitrates can be denitrified when soils are completely saturated, while very little denitrification occurs when soils are well drained. In 1995, Philipott *et al.* studied the role of the dissimilatory nitrate reductase in the competitive abilities of *Pseudomonas* strains, and proved that denitrification can contribute to the persistence and distribution of bacteria in fluctuating soil environments.

Nitrogen fertilization on sugarcane crops in Tucumán is carried out between October and November; consequently, the availability of nitrates is guaranteed during summer season, coinciding with the raining period. In this way, the amount of nitrates in soil and the input of rainfall water, plus the estival temperatures, do contribute to favor the denitrification process.

It is well known that soil texture (e.g. loamy in West area and clay-loam, silty-loam in East area, Table 1) and soil type (e.g. typic argiudoll-fluvenic in the West and typic ustifluvent-thaptoargic in the East, Table 1) are characteristics that affect moisture retention and aeration of the soil. Likewise, the clay content contributes to increase surface area and chemical properties of soils, affecting therefore the microbial activity. We speculate that in our case, these factors may surely

influence the differential occurrence of *Azospirillum* within the sugarcane cropping region studied. Eaton (2001) demonstrated that significant variations exist in the soil composition of different and closely situated habitats in the subtropical forest of Belize, associated with the relative richness of the vegetation. Considering this latter, as in our study the sampled area has only sugarcane plants, the variations observed in the MPN of denitrifiers would not be related to vegetation type but to environmental conditions.

Another important aspect that supports the influence of water (rainfall) in the distribution of *Azospirillum* in the sugarcane cropping region is the evapotranspiration process between the two areas under study. While in the West the mean annual evapotranspiration (potential) is 900 mm or less, in the East area it is 1046 mm. Comparing these values with the mean annual rainfall volumes in both areas, we can see a positive hydric balance (rainfall minus evapotranspiration) in the West area and a negative hydric balance in the East area. According to the higher occurrence of denitrifying isolates in the West area, we suggest that there is a clear influence of the rainfall and also soil type and texture, since the rate of drainage depends on these soil features. In a previous work, we observed a similar behavior about the environmental influence on the genetic diversity of *A. brasilense* isolates and hypothesized that the genetic diversity observed in different isolates can be a result of the selective pressure exerted by local chemical and physical-chemical properties of the soil over a certain period of time, giving place to a heterogeneity of environmental niches (Pedraza and Diaz-Ricci, 2003).

Although we have carried out PCR amplification of the *nirS* and *nirK* genes of all the isolates, regardless if they were denitrifiers or not, when nitrite reductase activity in semisolid NFb medium was assessed, only those that displayed *nir*⁺ activity showed amplification bands of the gene *nirS*. The latter confirms that the denitrifying isolates of *A. brasilense* belong to the *nirS* type of the nitrite reductase, which contains cytochrome *cd₁* as reported in other works (Bothe *et al.*, 1994; Braker *et al.*, 1998; Braker *et al.*, 2000; Kloos *et al.*, 2001). Our results also show that the variability observed in the *nir*⁺ activity of the isolates (number and size of bubbles) may be associated with the genetic variability detected in the *nirS* fragment through enzyme digestion.

According to our results, since *nir*⁺ isolates showed intragenic diversity within a functional nitrite reductase, we may speculate that the *nirS* fragment amplified does not correspond to a con-

served domain of the nitrate reductase enzyme. Furthermore, environmental differences in nutrient availability and humidity may also affect the kinetics of bacterial enzymes such as nitrite reductase. If we consider that these factors can work as selective agents on gene controlling enzyme systems, then we should accept that "a relationship does exist between various components of genetic diversity among populations of bacteria and variability associated with their environment" as suggested by McArthur *et al.* (1988).

RAPD analysis of the denitrifying *A. brasilense* isolates showed that these strains not only presented intragenic diversity within the *nirS* gene, but also differences at genomic level. Nevertheless, comparison of restriction profiles of the 16SrDNA fragment amplified by PCR of all isolates showed that they were similar, including the reference strain Sp7 of *A. brasilense*. However, from our results we can not infer that the genomic differences observed and presented in Fig. 4 can be associated with the denitrifying activity, nor to the variability detected in the gene *nirS*. The RAPD method amplifies at random DNA fragments that may not be necessarily the same corresponding to the *nirS* gene assessed in this study.

According to results presented in this work, effective non-denitrifying wild-type *Azospirillum* strains can be isolated from sugarcane roots that could be used as microbial inoculants as they are believed to be more beneficial for plant growth promotion. The knowledge of the ecology of denitrifiers (in this case, *Azospirillum* strains), represents a step forward to the rational utilization of the microbial diversity in agriculture.

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