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Genetic Stability of *Azospirillum brasilense* After Passing Through the Root Interior of Sugarcane

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Abstract

In this work we investigated the genetic stability of *Azospirillum brasilense* after passing four times through the root interior of sugarcane. This study is based on the observation of a wide genetic diversity by using the RAPD-PCR method of this species in the sugarcane cropping region of Tucumán, Argentina. Some phenotypic (e.g. motility, formation of a sub-superficial pellicle, shape, size, color of the colonies, production of melanine-like dark pigment) and genetic characteristics of a local endophytic strain of *A. brasilense* isolated from sugarcane root, used in this study, were analyzed. RAPD genomic fingerprints and plasmid profiles were used to detect genetic variations. No phenotypic change or genomic rearrangement was detected, according to the analysis of phenotypic characteristics, to the genomic DNA and plasmid profiles carried out of the original and reisolated strains of *Azospirillum*. After these results we conclude that the root interior of sugarcane constitutes a stable environment for *Azospirillum*, and preserves its characteristics as a strain under changing environmental conditions, when colonizing sugarcane roots.

Keywords: *Azospirillum brasilense*, genetic stability, plasmid profile, RAPD, sugarcane

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1. Introduction

When a strain of a microorganism proliferates clonally it is expected that the descendants would be genetically identical. Nevertheless, genetic variation within a bacterial clone (strain) is not a rare event (Blot, 1994) and microorganisms are subject to different types of modifications, generating a genetic diversity that can bring about the emergence and selection of new phenotypes (Schloter et al., 2000). Hence, the detection and determination of a strain variant can be a delicate and complex task. The successive cultivation of a strain under artificial laboratory conditions may promote genetic changes (Clerc et al., 1998). Several works indicate that when bacteria of *Rhizobium* are exposed to certain stressing conditions or genetic manipulation they can present genomic rearrangement (Berry and Atherly, 1984; Soberon-Chavez et al., 1986). Flores et al. (1988) also reported that *Rhizobium phaseoli*, under normal working conditions of laboratory (without stress), presented genetic rearrangement in the strains studied.

The genus *Azospirillum* belongs to a group of rhizosphere bacteria often referred to as plant growth-promoting rhizobacteria (Kloepper et al., 1989). They are free-living nitrogen-fixing bacteria closely associated with grasses (Steenhoud and Vanderleyden, 2000). Due to the increasing interest in making world agriculture sustainable, they are used as inocula for enhancing crop productivity and to replace chemical nitrogen fertilizers (Okon and Vanderleyden, 1997). 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; Bellone et al., 1997). Some strains of *Azospirillum* are endophytically localized in the plant roots. They can be found in intercellular spaces of the cortex, where an active exchange of nutrients and the nitrogen fixation process take place (Patriquin et al., 1983).

Therefore, it is hypothesized that a change in the ecological niche could affect the genomic stability of the bacteria. The latter would further explain the unexpected genetic diversity we detected in local strains of *A. brasilense*. RAPD analysis of many isolates from the sugarcane cropping area of Tucumán, Argentina, showed a large genetic diversity within the same species (*A. brasilense*), observing DNA polymorphisms with 12 primers used in the amplification; two identical DNA profiles were never found among the local isolates.

With the aim to investigate whether the DNA of *Azospirillum* remains stable or undergoes genomic rearrangement, some phenotypic and genetic characteristics of an endophytic strain of *A. brasilense* were studied, after four passages through the root interior of sugarcane plants.

2. Materials and Methods

Strain isolation

Eighteen locations within the sugacane cropping region in Tucumán, Argentina, were sampled by collecting sugarcane roots. Each sample consisted of six subsamples (10 g of roots) which were mixed together before assay. They were taken at random and kept at 4°C in sterile plastic bags until they were processed within 24 h of obtained. *Azospirillum* isolates were recovered from surface-sterilized roots of sugarcane, according to Döbereiner et al. (1995). Roots were vigorously washed with tap water and then dried between tissue paper. Root segments of 10–15 cm long were sealed with paraffin at their tips and then immersed in 1% chloramine-T dissolved in sterile water during 30 min. Then, roots were immersed for 10 min in sterile 0.05 M phosphate buffer (pH 7), and washed twice (10 min each) with sterile distilled water. Treated segments of the roots were cut into 1 cm long pieces with sterile scissors, transferred to N-free malate semisolid medium (NFb), (Döbereiner et al., 1995) and incubated 36–72 h at 30°C.

To check for surface contamination, root segments treated with 1% chloramine-T and with the tips sealed with paraffin were incubated in test tubes containing 12 ml NFb semisolid medium and imprinted on NFb solid medium supplemented with yeast extract (0.5 g l^{-1}). In all these cases no bacterial growth was observed.

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 72 hrs. After purification, the isolates were subjected to further characterization.

Microbiological characterization

The identification of the isolates was based on Gram staining, motility, cell shape observed by phase-contrast microscopy and colony morphology (Tarrand et al., 1978). The ability of the bacteria to grow on various carbon substrates was assayed in NFb medium replacing the malate source with other carbon substrates: maltose, sucrose, glucose, mannitol, N-acetylglucosamine, glycerol, D-fructose, citrate and myo-inositol. All carbon sources were sterilised by filtration and added to NFb medium after autoclaving. The assay was conducted with three replicates and reference strains were used as positive controls. Growth was considered positive when a typical pellicle below the surface of the medium was formed.

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 microbiological and molecular identification of the local isolates, were kindly provided by EMBRAPA-CNPq, Seropédica Km 47, R.J., Brazil.

Molecular identification

To identify the local isolates of *Azospirillum* a fragment of the gene 16S rDNA was analyzed, according to Grifoni et al. (1995). DNA used as template was obtained by thermal disruption of cells of different isolates and reference strains. Cell suspension (6 µl) of 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 reaction.

A fragment of the 16S rDNA gene from each isolate was amplified with specific primers (27f: 5' GAG AGT TTG ATC CTG GCT CAG 3' and 1495r: 5' CTA CGG CTA CCT TGT TAC GA 3') and the product digested with endonucleases: (5 U/µl) *Alu*I (Promega), *Bgl*I (Biolabs) and *Bsp*EI (Biolabs), according to the protocol of Grifoni et al. (1995). 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 volts/cm) in TBE 0.5X (Tris-borate-EDTA) running buffer (Sambrook et al., 1989). Gels were stained with ethidium bromide (0.5 µg/ml) for 30 min, washed three times with distilled water and photographed on UV analyzer with a Polaroid camera (Polaroid film 667).

Experiment 1: Genetic diversity of Azospirillum associated to sugarcane roots

The randomly amplified polymorphic DNA (RAPD-PCR) technique (Williams et al., 1990) was used to analyze the genetic diversity of 18 local isolates of *Azospirillum* associated to sugarcane roots. The samples for DNA extraction were obtained from suspension of cells grown overnight in liquid N-free malate medium (NFb) supplemented with 1 g l^{-1} NH_4Cl (Baldani and Döbereiner, 1980). Bacterial DNA was obtained as described by Sambrook et al. (1989) and purified with the Wizard™ Genomic DNA Purification Kit (Promega). The DNA was quantified with a Beckman 7000 spectrophotometer and polymerase chain reactions were carried out with 20 ng of DNA samples.

Reaction mixtures were prepared in 20 µl containing 0.5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} of each dNTP (deoxyribonucleotide), 0.2 µmol l^{-1} of random sequence 10-mer primers, 0.75 Units of *Taq* DNA polymerase (Promega) and 2 µl of *Taq* buffer (10×). Twelve primers were used: OPF and OPJ series (Operon Technologies) and series A of Biodynamics. The program used was:

denaturation, 30 s 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 volts cm^{-1} in a 1.5 % agarose gel in TBE 0.5X running buffer (Sambrook et al., 1989). Gels were stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$) for 30 min, washed three times with distilled water and photographed with a Polaroid camera (Polaroid film 667). RAPD experiments were repeated three times to avoid false results and to assure reproducibility. Negative controls, mix without DNA, were included in each experiment.

Experiment 2: Genetic stability of Azospirillum in sugarcane roots

Methodological strategy: An endophytic local strain of *Azospirillum*, isolated from sugarcane roots and characterized, as previously described, was used as inoculum. This strain was chosen for a particular phenotype that permits a fast and easy identification, namely, the production of a melanine-like dark pigment in N-free malate NFb semisolid medium (Baldani and Döbereiner, 1980). It was inoculated (10^7 CFU ml^{-1}) by watering the sugarcane vitroplants (variety CP 48-103) only once. The plants (five in each inoculation) were grown separately in sterile soil as substrate and placed into plastic pots disinfected with 7% NaOCl. Then, they were placed on disinfected plastic trays and were kept isolated in a breeding chamber (28°C, 70% of relative humidity and 16 h of photoperiod), to avoid cross contamination.

After 30 days from the first inoculation, samples of root where taken and treated under axenic conditions in order to recover the inoculated strain. The roots were separated in two groups. The first group was washed three times with tap water and then used for the isolation of bacteria that colonize externally the root surface. With the aim to obtain the endophytic bacteria, the second group was washed three times with tap water and surface sterilized (1% chloramine T), according to Döbereiner et al. (1995), as previously explained. In both cases, pieces of root (1 cm long) were placed in vials with N-free malate NFb semisolid medium (5 ml) and incubated 72 h at 30°C. After the isolation and characterization of the endophytic bacterium, this strain was used to inoculate a new batch of sterile plants as described above. The process of inoculation and reisolation of *Azospirillum* was performed every 30 days until the strain under study has passed four times through the root interior of sugarcane plants. During the growing period the plants received only sterile distilled water (when necessary).

Phenotypic evaluation of the strain: The motility, time to form the sub-superficial pellicle in NFb semisolid medium, shape, size and color of the

colonies on NFb solid media supplemented with yeast extract (0.5 g l^{-1}) and congo red (15 ml l^{-1} of a 1:400 aqueous solution), as well as the production of melanine-like dark pigment in NFb semisolid media were analyzed in each of the reisolates.

Identification of the strains by RAPD-PCR profiles: The RAPD-PCR method (Williams et al., 1990) was used to analyze the genetic stability of the strain under study. The DNA extraction and RAPD-PCR mixture and amplification conditions were as described before in this section. In this case, to evaluate the DNA profiles stability, the primers used were: (sequences from 5' to 3') OPJ-20: AAG CGG CCT C (Operon Technologies), A06 GAG TCT CAG G, A08 ACG CAC AAC C and A09 CTA ATG CCG T (Biodynamics). RAPD experiments were repeated three times to avoid false results and to assure reproducibility. Negative controls, mix without DNA, were included in each experiment.

Plasmid profiles: The strains (original and reisolates) were grown overnight in LB medium (Sambrook et al., 1989) in a water bath shaker at 37°C. The presence of plasmids was detected using the in-well lysis method described by Eckhardt (1978) and modified by Hynes and McGregor (1990). Cells of an overnight culture (0.1 ml) grown in LB medium (OD_{600} = 1) were washed twice with cold 0.3% sarkosyl (4°C) in Tris-borate buffer, followed by centrifugation (14,000 rpm for 7 min.). The cell pellet was gently suspended to avoid a premature cell disruption. After washing, the pellet was suspended in 0.02 ml of lysis solution (sucrose 10%, RNase 10 µg ml^{-1} in TBE and lisozyme 1 µg ml^{-1}) and mixed with 0.03 ml of loading buffer (Sambrook et al., 1989) just before loading the gel.

The gels (0.7% agarose), supplemented with SDS (1%) were prepared and run in TBE 1X buffer (Sambrook et al., 1989). Gels were run at 0.7 V cm^{-1} during 1 h, followed by 1.40 V cm^{-1} for 2 h and 2.81 V cm^{-1} for further 3 h. DNA was stained for 30 min in ethidium bromide (0.5 µg ml^{-1}), washed in distilled water for 50 min, and photographed with Polaroid 665 film at 320 nm UV.

In order to test the influence of a different biological environment, plant species and photosynthetic metabolisms (e.g. C-4 for sugarcane and C-3 for strawberry) an experiment similar that the one described above to test the genetic stability of *Azospirillum* in sugarcane roots was carried out with strawberry vitroplants, variety Chandler.

3. Results

Growth of the isolates in NFb semisolid medium was observed in all samples collected from surface-sterilised 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

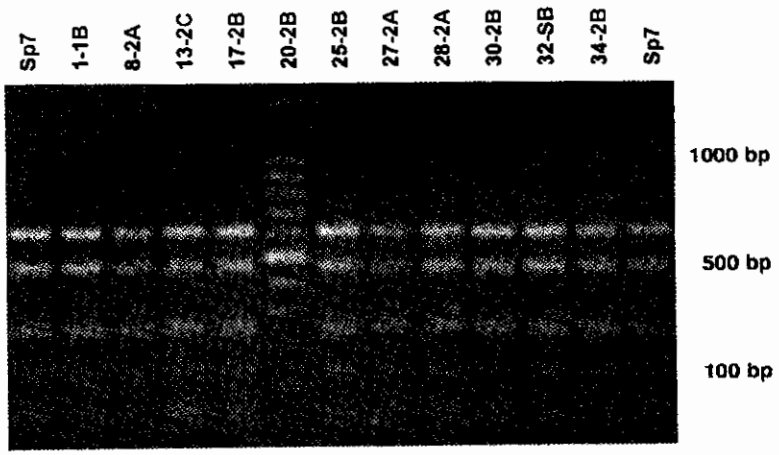

Figure 1. Electrophoresis of amplified 16SrDNA fragments digested with *Alu*I. The profiles correspond to local *A. brasilense* isolates from the sugarcane cropping region of Tucumán, Argentina. Sp7 is the strain of reference for *A. brasilense*. WM: molecular weight marker Ladder 100 bp (Promega).

dry consistency, with round form and wrinkled edges. The use of different carbon sources was positive for glycerol and D-fructose. According to these characteristics and comparison with the positive controls we identified the species *A. brasilense*. These results were confirmed by restriction profiles of a 1450 bp fragment of the gene 16SrDNA amplified by PCR and digested with the endonucleases *Alu*I, *Bgl*I and *Bsp*EI, using the strain *A. brasilense* Sp7 as positive control (Fig. 1).

Experiment 1: Genetic diversity of Azospirillum associated to sugarcane roots

Amplification profiles generated by the RAPD technique showed different patterns for the isolates assessed. The number and size of amplified products (bands) ranged from 1–8 and 200–2000 base pairs, respectively (see Fig. 2). As shown in Fig. 2, RAPDs profiles of *A. brasilense* strains displayed wide intra-species diversity with very few monomorphic bands and many polymorphic bands indicating a rich genomic diversity within the species. In Fig. 2 we can also see that the RAPD technique could easily discriminate among the 18 strains with only few primers (A-06, A-08, OPF-01 and OPJ-11). Only two strains, the ADU3 and RAN1 presented a relatively similar band pattern (Fig. 2C) and it was observed only with the primers OPF-01 and OPJ-10. The rest of the strains presented rather dissimilar DNA fingerprint regardless the primer used in the experiment.

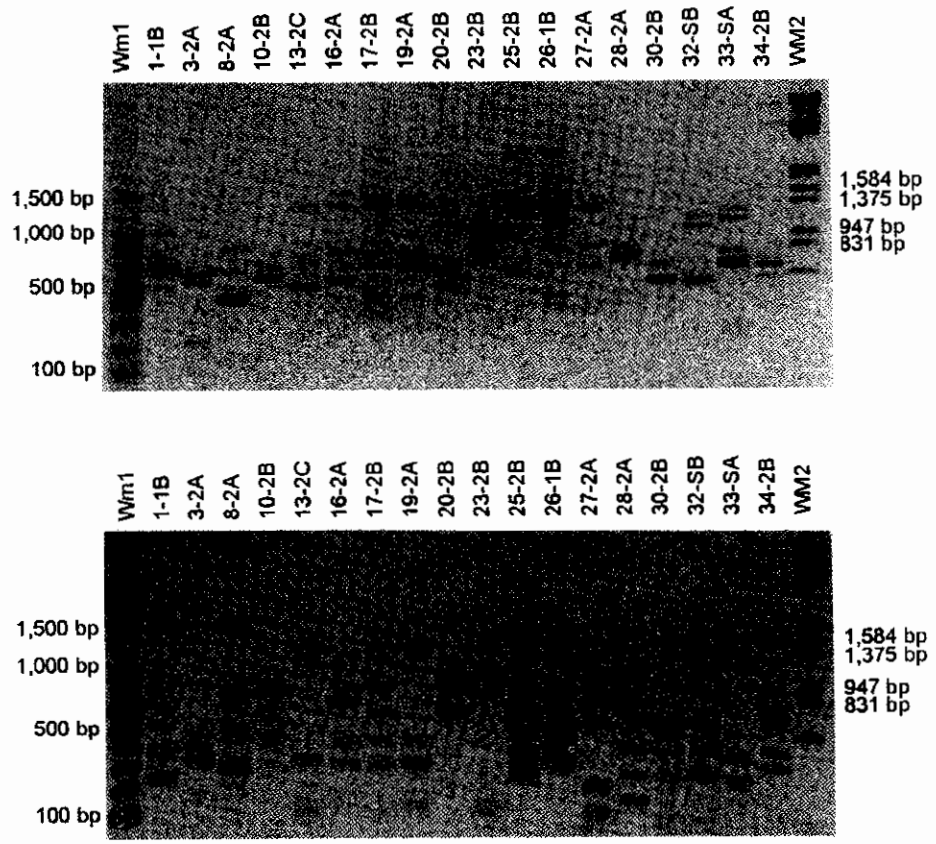

Figure 2. RAPD profiles of *A. brasilense* local strains isolated from the root interior of sugarcane (negative image of horizontal gel electrophoresis). A, B, C and D correspond to the amplifications with primers A-08, A-06, OPF-01 and OPJ-11, respectively. M1: molecular weight marker Ladder 100 bp (Promega). M2: molecular weight marker Lambda *Eco*RI-*Hind*III (Promega).

Experiment 2: Genetic stability of Azospirillum in sugarcane roots

Endophytic and surface colonizing bacteria were isolated and characterized from roots, after 30 days of inoculation of *in vitro* sterile sugarcane plants in every cycle.

Colonies of all isolates growing in NFb solid medium supplemented with yeast extract and congo red had the following characteristics after 96 h of

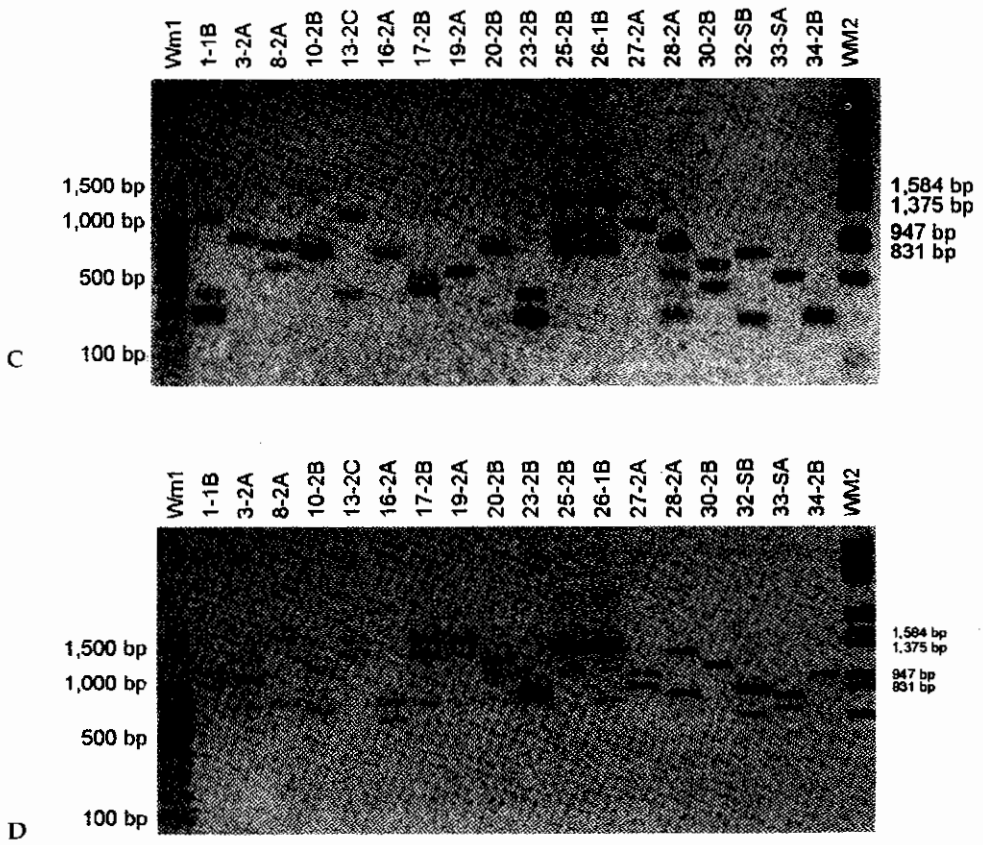

Figure 2. Continued.

incubation at 30°C: scarlet color, dry consistency, diameter of 1.5 to 2.0 mm, round form and wrinkled edges.

The time elapsed to form the sub-superficial undulated thin pellicle in NFb semisolid media, typical of *Azospirillum*, was within 24 h of cultivation at 30°C. After this period, the inverted bell-shape pellicle evolved to a white dense pellicle located 1 mm below the medium surface.

The motility of the original strain and all the isolates was the same as observed microscopically in fresh cultures (e.g. rods with active movements characteristic of *Azospirillum* spp.).

The formation of the dark brown melanine-like pigment observed in colonies of the strain used, is a distinctive feature of this strain and constitutes a very

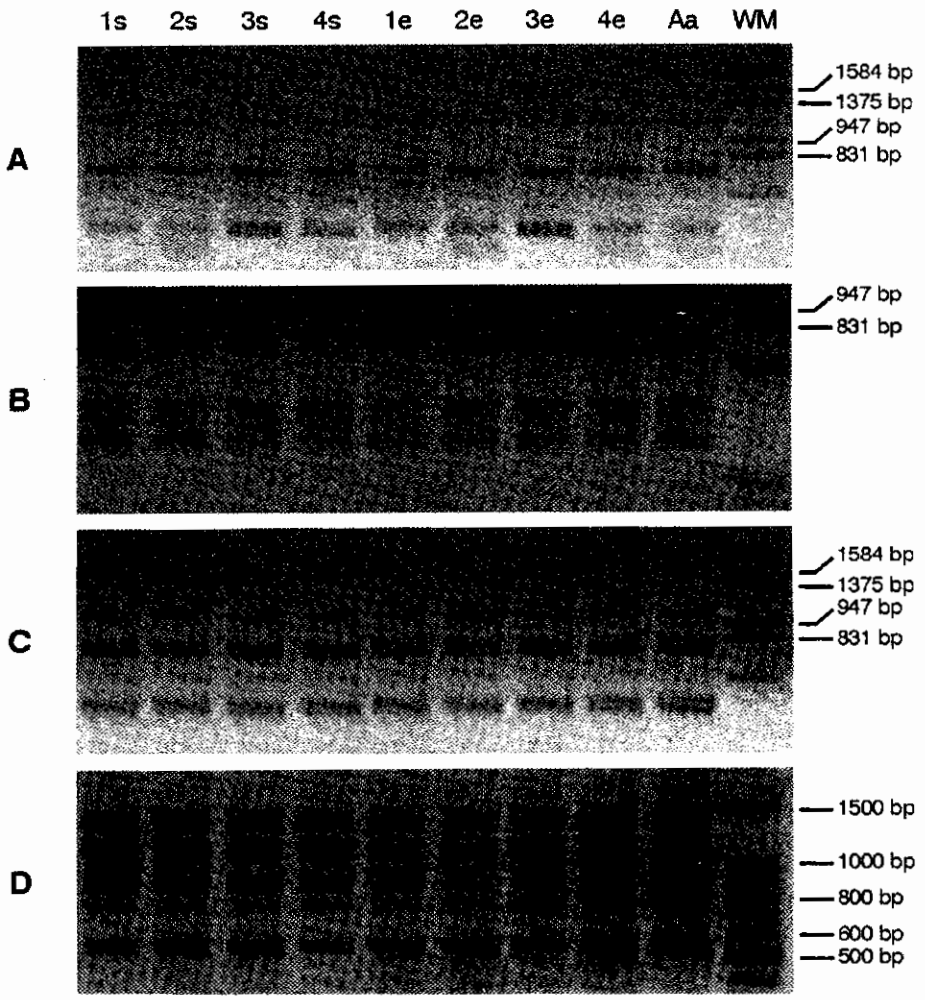

Figure 3. RAPD profiles of *A. brasilense* (negative image of horizontal gel electrophoresis). A, B, C and D correspond to the amplifications with primers A06, A08, A09 and OPJ20, respectively. 1s, 2s, 3s and 4s are the profiles of the 1st, 2nd, 3rd and 4th *A. brasilense* root surface colonizing reisolates. 1e, 2e, 3e and 4e are the profiles of the 1st, 2nd, 3rd and 4th *A. brasilense* root endophytic reisolates, after inoculation in sugarcane. Ab: original *A. brasilense* strain used in the first inoculation. WM: molecular weight markers; Lambda *Eco*RI-*Hind*III (Promega), for A, B, C and Ladder 100 bp (Promega) for D.

easy and convenient phenotypic character to follow, as has been already reported in aging cultures of *A. brasilense* (Sadasivan and Neyra, 1987). The

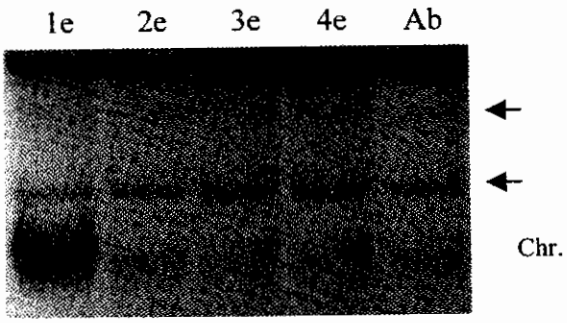

Figure 4. Plasmid profiles of *A. brasilense* (negative image of horizontal gel electrophoresis). Lines 1e, 2e, 3e and 4e are the profiles of the 1st, 2nd, 3rd and 4th *A. brasilense* root endophytic reisolates, after inoculation in sugarcane. Ab: original *A. brasilense* strain used in the first inoculation. Arrows indicate the position of the plasmids. "Chr." indicates the chromosomal DNA.

production of a dark brown pigment in NFb semisolid media was observed after 10 days of incubation in each of the reisolates.

These results showed that the phenotypic characteristics of the strain remained unchanged during the study.

RAPD profiles generated with the four random decanucleotide primers selected, revealed that there was no change in DNA fingerprints of all reisolates. The DNA patterns were identical in number and size of the bands amplified, regardless whether they were from endophytic or root surface colonizing bacteria (Fig. 3).

Fig. 4 shows that the plasmid profiles of the recovered endophytic strains were the same as the original strain used in the first inoculation. The number and size of plasmids agreed with previous reports about the presence of plasmids in *A. brasilense* (Plazinski et al., 1983; Onyeocha et al., 1990).

Identical results were obtained from the experiment carried out with strawberry plants, under the same experimental conditions used for sugarcane (data not shown).

4. Discussion

Results obtained in Experiment 1 with RAPD analysis of strains isolated from surface-sterilised sugarcane roots show that there is a wide genetic diversity among isolates within different locations of the sugarcane cropping region of Tucumán, Argentina. Although all classical and molecular procedures

used for the identification of the local isolates confirmed the identity of *A. brasilense,* we have observed DNA polymorphism with 12 different 10-nucleotide random primers (Fig. 2). This finding conducted us to postulate whether the sugarcane root by itself would be a source of genetic variation for endophytic *Azospirillum.*

Simple phenotypic analysis is limited as the whole potential information of a genome is never expressed because gene expression is strongly influenced by environmental conditions (Rosselló-Mora and Amann, 2001). However, in Experiment 2, we have used some bacterial phenotypic features because they were analyzed under the same controlled environmental conditions, ensuring in this way reliable and comparable observations. Results of our study showed that there is no evidence of phenotypic changes of the strain inoculated into sugarcane plants during the successive passages through the root interior.

With the aim to study possible changes that may have occurred at the DNA level and would not be detectable or inferred with simple phenotypic analyses we have carried out a genetic analysis of two types: genomic DNA fingerprinting by RAPD-PCR and plasmid profile by in-well mild lysis.

Genomic DNA fingerprinting permits the detection of genomic rearrangement, intraspecific diversity and to reveal differences among close relative organisms (Vandamme et al., 1996). RAPD-PCR analysis has been successfully used for the identification of *Bifidobacterium longum* SBT2928 recovered after intestinal passage (Fukiwara et al., 2001) and also for the assessment of the genetic stability of *Shewanella putrefaciens* with RAPD-PCR genotyping technique using three random decanucleotide primers (Ziemke et al., 1997). We have used the RAPD technique to evaluate DNA fingerprints of all *Azospirillum* strains isolated to bring additional support to the phenotypic analyses. By analyzing the genomic DNA profiles generated by RAPD with four random decanucleotide primers we did not detect any genetic change nor genomic rearrangement (Fig. 1).

Likewise, plasmid profiles of the endophytic recovered strains and the original inoculum, showed no change in the extrachromosomal DNA (Fig. 2). The similitude of the plasmid profiles were enough to further support the identity of the strain used in the starting inoculum with the strains reisolated from sugarcane roots during the study.

All these results indicate that the successive passage of this strain of *A. brasilense* through the interior of roots do not induce any phenotypic nor genetic change. That is, the metabolic stress that *Azospirillum* must overcome when moving from the ecological niche of the soil to the environment of the root surface or the microaerobic root interior found in sugarcane does not induce any change. Furthermore, we suggest that there was no metabolic influence in this behavior as identical results were obtained in this study when the same *A. brasilense* strain was inoculated into strawberry roots (data not shown).

Our results in Experiment 2 also show that the physiological stress these bacteria must bear during the process of recovery and successive cultivation under laboratory conditions did not cause any observable pheno- or genotypical change, at least under these experimental conditions, in contrast to what was reported by Berry and Atherly (1984), Soberon-Chavez et al. (1986), Flores et al. (1988) and Clerc et al. (1998) in *Rhizobium* and other microorganisms.

These experiments demonstrate the genetic stability of *Azospirillum* under changing environmental conditions and specially when colonizing sugarcane roots. The latter may have interesting implications when using this bacterium as crop inoculant for it would keep unchanged the beneficial agronomic characteristics of *Azospirillum* during its association with the plant. From ecological stand point, we can also speculate that the genetic stability shown by *Azospirillum* would provide further advantage for the soil colonization through horizontal endophytic dissemination within sugarcane plants preserving the original genetic information.

Finally, according to the results obtained, we conclude that since *Azospirillum* proved to be genetically stable during the passage through the interior of sugarcane roots, at least in short term experiments, the origin of the large genetic diversity observed in Experiment 1 with strains of *A. brasilense* remains unclear. We may speculate that the large genetic diversity observed in different isolates is due to the selective pressure exerted by local chemical and physical-chemical properties of the soil over a certain period of time, resulting in heterogeneity of environmental niches. On the other hand, endophytic cells living in sugarcane roots might be buffered from environmental changes and one might expect that there would be less selective pressure resulting in observable phenotypic and genetic changes.

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