REGULAR ARTICLE

Natural occurrence of *Azospirillum brasilense* in strawberry plants

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Abstract *Azospirillum* species are free-living nitrogen-fixing bacteria commonly found in soil and in association with roots of different plant species. For their capacity to stimulate growth they are known as plant growth-promoting bacteria (PGPB). In this work, we demonstrate the natural occurrence and colonization of different parts of strawberry plants by *Azospirillum brasilense* in the cropping area of Tucumán, Argentina. Although bacteria isolations were carried out from two strawberry cultivars, e.g., Camarosa and Pájaro, attempts were successful only with the cultivar Camarosa. Whereas different strains of *Azospirillum* were isolated from the root surface and inner tissues of roots and stolons of the cultivar Camarosa, we have not obtained *Azospirillum* isolates from the cultivar Pájaro. After microbiological and molecular characterization (ARDRA) we determined that the isolates belonged to the species *A. brasilense*. All isolates showed to have the capacity to fix nitrogen, to produce siderophores and indoles. Local isolates exhibited different yields of indoles production when growing in N-free NFb semisolid media supplemented or not with tryptophan (0.1 mg ml^{-1}). This is the first report on the natural occurrence of *A. brasilense* in strawberry plants, especially colonizing inner tissues of stolons, as well as roots. The local isolates showed three important characteristics within the PGPB group: N_2-fixation, siderophores, and indoles production.

Keywords *nif*D-gene · N_2-fixation ·
Phytohormones · Plant growth-promoting bacteria ·
Siderophores

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Introduction

Strawberries are cultivated in different parts of the world, including tropical, subtropical, and temperate areas. Besides its commercial interest, this crop is of great social concern due to the high demand of workers required for its production and processing at the field, packing, and industry. The world production of strawberry is estimated in three million tonnes per year and it has increased in the last two decades. Argentina produces strawberry during the 12 months of the year, being the province of Tucumán one of the most important producers (Pérez and Mazzone 2004).

Although this crop is frequently grown in soils with good natural fertility, chemical fertilizers, mainly nitrogen, are applied at levels over 290 kg ha^{-1} year^{-1} to improve yields. The high rate of application of fertilizers and pesticides have negative and unpredictable effects in the environment, contributing to the contamination of soils, water, and natural areas, casting serious risks to human and animal health. In addition, developing countries have to face the problem of higher costs for technology and chemical utilization. An interesting option for the substitution of nitrogen fertilizers could be the exploitation of plant growth-promoting bacteria (PGPB), such as bacteria of the genus *Azospirillum*, capable of affecting growth and yield of many plant species, several of agronomic, and ecological significance.

Azospirillum species are free-living nitrogen-fixing bacteria commonly found in soils and in association with roots of plants, including important agricultural crops such as maize, wheat, pearl millet, sorghum, and legumes (Baldani et al. 1983; Bashan et al. 2004; Burdman et al. 1997; Pacovsky 1990; Saubidet et al. 2002; Tien et al. 1979). Rhizosphere colonization by *Azospirillum* species has been shown to stimulate growth of a variety of plant species, although the basis for this stimulation is not clear at present. The principal hypothesis about its growth promotion capacity, seems to lie in the capacity to produce various phytohormones (e.g., indolic compounds such as indole-3-acetic acid and indole butyric acid) that improve root growth, absorption of water and minerals that eventually yield larger, and in many cases, more productive plants (Dobbelaere et al. 2001; Martínez-Morales et al. 2003). Initially, it was assumed that the nitrogen fixing capacity of *Azospirillum* was the principal mechanism by means of which *Azospirillum* could bring about the plant growth promotion, but at present this feature is controversial (for reviews: Bashan and Levanony 1990; Bashan and Holguin 1997; Bashan et al. 2004). The production of siderophores by *Azospirillum* is another feature that could contribute to its proliferation in an iron-poor environment of host tissues (Shah et al. 1992). Bashan and Levanony (1990) have shown that various mechanisms participate simultaneously, coordinate and cooperatively in the plant-bacteria association, causing the plant growth enhancement when they take place under appropriate environmental conditions.

Although the literature is abundant in industrial crops, little attention has been paid to the occurrence of PGPB in fruit crops. Few reports were found showing the presence of *Azospirillum* species in the rhizosphere of various fruit crops, including banana and pineapple plants (Ghai and Thomas 1989; Subba Rao 1983; Weber et al. 1999). In this article, we report the natural occurrence and colonization of different parts of strawberry plants grown in Tucumán, Argentina by *Azospirillum brasilense*, showing three important characteristics within the PGPB group: N$_2$-fixation, siderophores, and indoles production.

Materials and methods

Plant cultivars and tissue sampling

Plants of strawberry (*Fragaria × ananassa*) cultivars ''Camarosa'' and ''Pájaro,'' both with photoperiod of short days, were collected from the experimental field of the Faculty of Agronomy and Zootechnics of the National University of Tucumán, located in the province of Tucumán, Argentina (26 47′S−65 16′W). Samples of roots, leaves, petioles, and stolons (Fig. 1) were collected during active growth of the plants (60 days after transplanting). About 10 g of roots, leaves, petioles, and stolons were collected at random from 20 plants of each cultivar, in different parts of the plantation and pooled before assay. They
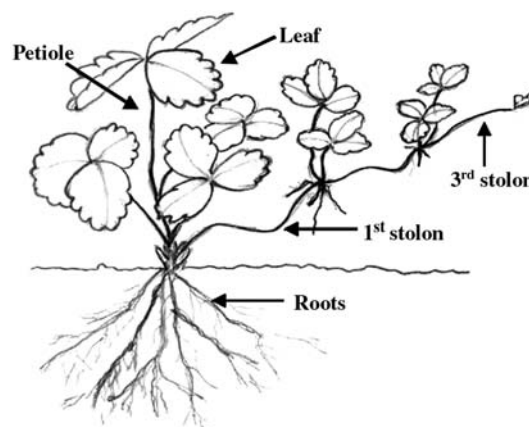

Fig. 1 Scheme of mother and daughter strawberry plants connected through stolons. *Arrows* indicate places where samples were taken to isolate *Azospirillum*

were kept in sterile plastic bags and immediately processed in laboratory. In strawberry plants, a stolon is a branch that emerges from the crown and grows horizontally above the medium and produces roots and shoots at nodes. When studying *Azospirillum* translocation through inner tissues and its capacity to colonize uninoculated plants, we have considered the first and third stolons extending from the original mother plants to the new daughter plants, isolated from the soil (Fig. 1).

Strain isolation, enumeration, and identification

Azospirillum isolates were recovered from washed and surface-sterilized roots of strawberry according to Döbereiner et al. (1995). Roots were vigorously washed with tap water and then dried between tissue paper. To obtain *Azospirillum* that colonize the root surface (RLC), washed roots were cut into 1 cm long pieces, transferred to N-free malate semisolid (NFb) and LGI media (Döbereiner et al. 1995) and incubated 36–72 h at 30°C. The NFb media composition is as follows: 5.0 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2 \times 2H_2O$, 2.0 ml micronutrients solution (0.04 g $CuSO_4 \times 5H_2O$, 1.20 g $ZnSO_4 \times 7H_2O$, 1.40 g H_2BO_3, 1.00 g $NaMoO_4 \times 2H_2O$, and distilled water to 1,000 ml), 2.0 ml bromothymol blue (0.5% w/v in 0.2 N KOH), 4.0 ml FeEDTA (1.64% w/v, aqueous), 1.0 ml vitamins solution (10 mg biotin, 20 mg piridoxal-HCl, and distilled water to 100 ml), 4.5 g KOH, and distilled water to 1,000 ml, pH 6.8, adjusted with NaOH. For semisolid medium 1.75 g agar per liter. The LGI medium composition is as follows: 5.00 g cane sugar, 0.2 g K_2HPO_4, 0.6 g KH_2PO_4, 0.2 g $MgSO_4 \times 2H_2O$, 0.02 g $CaCl_2 \times 2H_2O$, 0.002 $Na_2MO_4 \times 2H_2O$, 5.0 ml bromothymol blue, 4.0 ml FeEDTA, 1.0 ml vitamins solution, and distilled water to 1,000 ml, pH 6.0, adjusted with H_2SO_4. For semisolid medium 1.75 g agar per liter. To obtain azospirilla that colonize internally the root tissues (REC), root segments of 8–10 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 NFb and LGI media (Döbereiner et al. 1995), and incubated 36–72 h at 30°C. Surface contamination tests were carried out with root segments treated with 1% chloramine-T, tips sealed with paraffin, incubated firstly in test tubes containing 12 ml NFb semisolid medium (36–72 h at 30°C) and then imprinted on NFb solid medium supplemented with yeast extract (0.5 g l^{-1}). In all these cases no bacterial growth was observed after 5 days of incubation at 30°C.

The procedure previously described to isolate *Azospirillum* from inner root tissues was used also to isolate them from petioles and stolons (PEC). In the case of leaves, they were vigorously washed with tap water, then disinfected with ethanol 95 and 70%, cut into pieces of 0.25 cm^2, transferred to NFb and LGI semisolid media (Döbereiner et al. 1995), and incubated 36–72 h at 30°C.

From all plant tissues sampled, the MPN per gram fresh root, leaf, petiole, and stolon was determined according to Döbereiner et al. (1995), using the McCrady table for three replicates in NFb and LGI semisolid media. About 10 g of different plant tissue was macerated in 90 ml of a sucrose water solution (4%, w/v). Then, serial dilutions were made in the same solution. 0.1 ml from each dilution was placed in the middle of the growth culture medium (three vials per dilution) and incubated 48–36 h at 30°C. Presence or absence of a pellicle in the growing medium was determined.

Cultures forming a typical white pellicle below the surface of the medium were streaked out on Petri dishes containing NFb or LGI solid medium supplemented with yeast extract (0.5 g l^{-1}). Individual colonies were newly transferred into vessels with NFb or LGI semisolid medium and incubated at 30°C for 72 h. After purification, 20 isolates were taken at random for further identification analysis.

The identification of isolates was based on microbiological, biochemical, and molecular tests specifically reported for *Azospirillum* species (Tarrand et al. 1978; Döbereiner et al. 1995; Grifoni et al. 1995). The strains *A. brasilense* Sp7 (ATCC 29145) and Sp245, *Azospirillum lipoferum* Sp59 (ATCC 29707), *Azospirillum amazonense* Y1 (ATCC 35119), *A. irakense*, and *A. halopraeferens* used as references were kindly provided by EMBRAPA-Agrobiologia, Seropédica Km 7, R.J., Brazil.

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 semisolid 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 sterilized by filtration and added to NFb medium after autoclaving. Assays were conducted with three replicates including reference strains. Growth was considered positive when a typical pellicle bellow the surface of the medium was formed.

Molecular characterization

To identify the local isolates of *Azospirillum* a fragment of the gene 16S rDNA 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 sterile double-distilled water, boiled at 95°C for 10 min, and cooled down at room temperature. About 1 μl of this suspension was used in the PCR reaction.

A fragment of the 16S rDNA gene from each isolate was amplified with primers 27f and 1495r (synthesized by Biodynamics, Buenos Aires, Argentina) according to Grifoni et al. (1995). The amplification products (15 μl and 1.5 μg of DNA) were digested with 5 U μl^{-1} of the endonuclease *Alu*I (Promega, Madison, WI, USA). 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 Tris–borate–EDTA (TBE) 1× 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.

Potential N$_2$-fixing capacity determination

To probe the potential N$_2$-fixing capacity of the local bacterial isolates we used a molecular approach. It consisted in the PCR-amplification of a 710 bp fragment of the gene *nif*D, essential in the biological N$_2$-fixing process, using the primers and conditions prescribed by Potrich et al. (2001). The capacity of the local isolates to have a diazotrophic growth was observed performing successive replications from one N-free NFb semisolid medium to others new ones (no less than ten times). *A. brasilense* Sp7 was used as positive control and *Escherichia coli* MC4100, grown in LB media, as negative (Sambrook et al. 1989).

Acetylene reduction assay (ARA)

The nitrogenase activity Acetylene reduction assay (ARA) was carried out by duplicate in 50 ml vials containing 25 ml of N-free malate NFb semisolid medium. Isolates from strawberry plants were grown in this medium for 48 h at 30°C. Each vial was sealed with rubber stopper and the head space (25 ml) was injected with 10% (v/v) acetylene. Gas samples (0.2 ml) were removed after 1 h and assayed for ethylene production with a gas chromatograph (Carle Analytical Gas Chromatograph model 311), using a Porapak-N column (5 m × 0.5 mm, 35°C) and a hydrogen flame ionization detector. Values were expressed as nmoles C$_2$H$_4$ h^{-1} culture^{-1}. Results were subjected to ANOVA and LSD ($P = 0.05$) analysis with the Statistix Analytical Software 1996 for Windows.

Production of siderophores

The chrome azurol sulfonate (CAS) assay was used to determine production of siderophores (Schwyn and Neilands 1987). Bacteria were spot inoculated by triplicate in Petri dishes containing NFb solid medium, without bromothymol blue, and supplemented with 1% NH$_4$Cl and CAS. They were incubated at 30°C for 96 h.

Total indoles production

Total indoles production was determined by a colorimetric method (Glickmann and Dessaux

1995) in N-free malate semisolid medium supplemented or not with tryptophan (Trp) (0.1 mg ml^{-1}) according to the following procedure. Bacteria were streaked out on NFb solid medium, supplemented with yeast extract (0.5 g l^{-1}) and Congo Red (15 of a 1:400 aqueous solution). After 1 week of incubation at 30°C, one colony of ∼0.5 mm of diameter of each bacterial isolate was transferred to NFb semisolid medium without bromothymol blue. Cultures were incubated at 30°C during 1 week and then centrifuged (5,000×g, 10 min) to obtain the supernatant. Indoles production was determined with Salkowski reagent. The reaction was carried out with 500 μl of supernatant and 500 μl of Salkowski reagent, followed by incubation in the dark at room temperature during 30 min. Total indoles were determined spectrophotometrically at 540 nm, using authentic indole acetic acid as standard. The test was carried out by triplicate in each case, e.g., growth medium supplemented or not with Trp and results were subjected to ANOVA and LSD (P = 0.05) analysis with the Statistix Analytical Software 1996 for Windows.

Protein quantification

Protein concentration of the local isolates was determined by the method of Bradford (1976) using bovine-serum albumin as standard.

Results

Azospirillum growth was observed only in NFb culture medium from washed root, surface sterilized roots, and first stolons coming from cultivar Camarosa. Attempts to obtain isolates from the cultivar Pájaro were always unsuccessful, although the isolation procedure was repeated several times under these experimental conditions. In Table 1, we present data based on plant tissues where *Azospirillum* was isolated from and the MPN per g plant material. The highest number was obtained from sterilized roots (2.5 × 10^4 g^{-1} fresh root) whereas the lower from the first stolons (2 × 10^2 g^{-1} fresh stolon). We did not obtain *Azospirillum* from leaves, petioles, and third stolons of strawberry plants neither in NFb nor LGI semisolid media.

Bacterial isolates coming from positive growth in NFb semisolid medium showed Gram-negative staining, motility, and S-shape cells observed by phase-contrast microscopy. Colonies formed on NFb solid medium supplemented with yeast extract and Congo Red showed dry consistency, red color, with round form and wrinkled edges. The use of different carbon sources was positive for glycerol, D-fructose, sodium succinate, sodium lactate, and malic acid. According to these characteristics and comparison with the positive controls we identified the species as *A. brasilense*. These results were confirmed by restriction profiles of a 1,450 bp fragment of the gene 16S rDNA amplified by PCR and digested with the endonuclease *Alu*I, using the strains Sp7 and Sp245 of *A. brasilense* as positive controls and *A. lipoferum* Sp59, *A. amazonense* Y1, *A. irakense*, and *A. halopraeferens* to differentiate with the first two ones (Fig. 2).

To determine the potential N$_2$-fixing capacity of the isolates we used a molecular approach. A 710 bp fragment of the gene *nif*D was amplified by PCR in all the local isolates and reference strain (Sp7). Our results agree with results obtained by Potrich et al. (2001) (Fig. 3).

The nitrogenase activity of the local isolates was assessed by measuring the reduction of acetylene to ethylene, observing different values among the local isolates. REC3 showed the highest value (115.0 ± 5.2 nmoles C$_2$H$_4$ h^{-1} culture^{-1}) and RLC6 the lowest (41.5 ± 7.6 nmoles C$_2$H$_4$ h^{-1} culture^{-1}) (Fig. 4). A global evaluation of ARA values indicate

Table 1 Plant tissues where *Azospirillum* was found and MPN

Cultivar	Leaf	Petiole	Washed root	Sterilized root	First stolon	Third stolon
Pájaro	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Camarosa	n.d.	n.d.	+ (4.5 × 10^3)	+ (2.5 × 10^4)	+ (2 × 10^2)	n.d.

Values between parentheses represent MPN values expressed as bacteria per gram of root and stolons, respectively

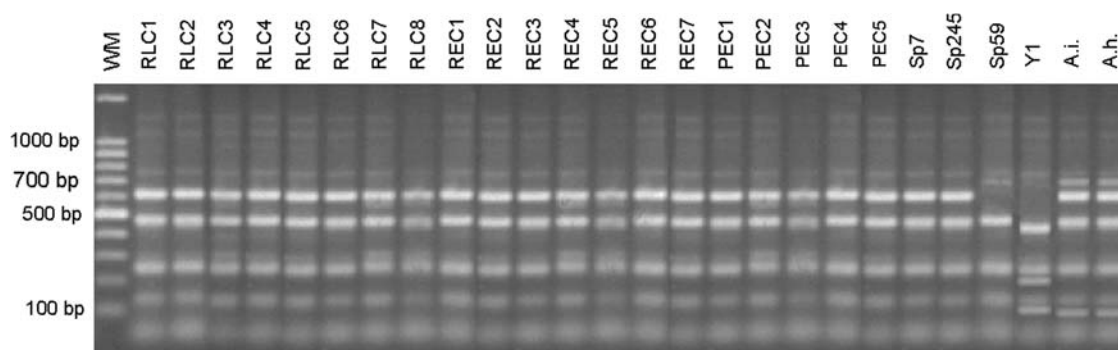*n.d.* not detected, + positive isolation of *Azospirillum* in NFb medium

Fig. 2 Electrophoretic patterns of amplified 16S rDNA fragments digested with *Alu*I. The profiles correspond to local *A. brasilense* isolates from washed roots (RCL1 to 8), surface sterilized roots (REC1 to 7), and stolons (PEC1 to 5) of strawberry plants. Sp7 and Sp245: *A. brasilense*, Sp59: *A. lipoferum*, Y1: *A. amazonense*, A.i.: *A. irakense*, and A.h.: *A. halopraeferens*. *WM*: molecular weight marker Ladder 100 bp (Promega)
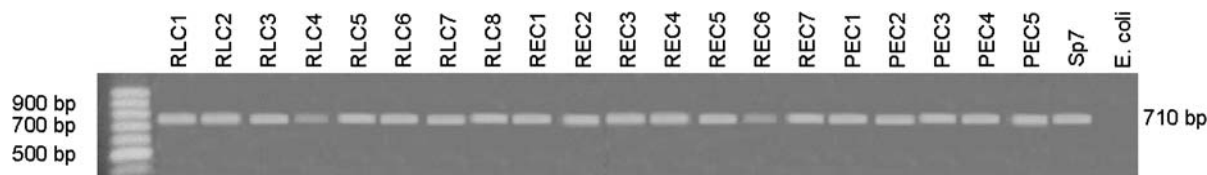

Fig. 3 Electrophoretic patterns of amplified 710 bp *nif*D fragments. The profiles correspond to local *A. brasilense* isolates from washed roots (RCL1 to 8), surface sterilized roots (REC1 to 7), and stolons (PEC1 to 5) of strawberry plants. Sp7: reference strain of *A. brasilense* and *E. coli*: strain MC4100. First line without label: molecular weight marker Ladder 100 bp (Promega)
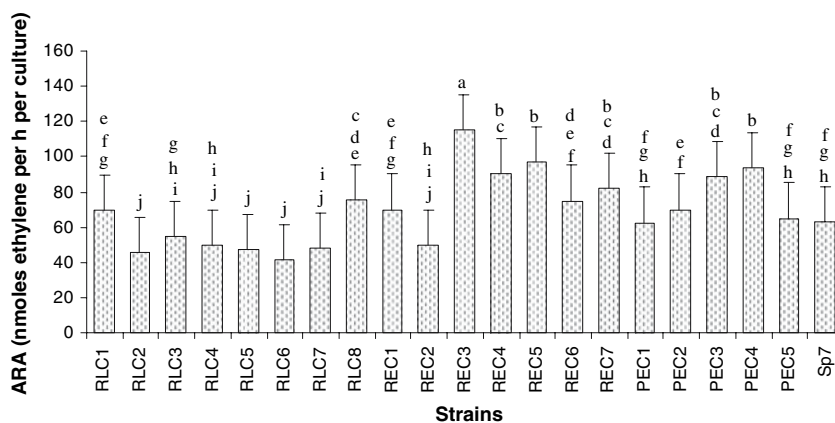

Fig. 4 Ethylene production of different *A. brasilense* strains isolated from strawberry plants grown in N-free NFb semisolid medium Isolates RLC1 to 8: correspond to strains isolated from washed roots; REC1 to 7: strains isolated from surface sterilized roots; PEC1 to 5: strains isolated from inner tissues of first stolons. Sp7: reference strain of *A. brasilense*. Data are the means of two determinations and the *error bars* indicate SD. *Different letters* indicate significant differences at $P = 0.05$

that strains coming from surface sterilized roots and stolons show slightly higher nitrogenase activity than those obtained from washed roots ($P = 0.05$).

Production of siderophores was observed in all the isolates by a color change from blue to orange on the culture media, forming clear zones around the

colonies. The results were qualitatively distinct in terms of the size of the clear zones formed.

Considering the phytohormone production as one of the features for *Azospirillum* as PGPB, we determined the biosynthesis of total indoles in all local isolates in N-free NFb semisolid medium, without bromothymol blue, and supplemented or not with Trp. Figure 5A, B shows values of total indoles detected in isolates from washed and surface sterilized roots as well as from inner tissues of stolons. In that figure, we can see that the presence of Trp has a significant incidence in the biosynthesis of total indoles in all the isolates, including the reference strain Sp7 of *A. brasilense* (Fig. 5B), a well documented producer of indolic compounds (Baca et al. 1994; Martínez-Morales et al. 2003; Pedraza et al. 2004). In general, a variation of values of indolic compounds among isolates was observed, being the strains REC3 (0.49 ± 0.01 µg mg protein^{-1}) and PEC1 (1.78 ± 0.02 µg mg protein^{-1}) the higher producers without and with addition of Trp as precursor, respectively. On the other side, the strain

RLC5 was the lowest producer in absence (0.19 ± 0.01 µg mg protein^{-1}) or presence of Trp (0.46 ± 0.01 µg mg protein^{-1}) in the growing medium (Fig. 5A, B).

Discussion

In this work, we confirm the associative nature of *Azospirillum* colonizing the surface and inner tissues of strawberry roots as well as inner tissues of stolons. The isolation of *Azospirillum* from different parts of strawberry plants demonstrates the rhizosphere and endophytic nature of this genus, confirming what was already reported by others, e.g., maize, sorghum, and wheat (Baldani et al. 1983; Baldani et al. 1986; Umali García et al. 1980; Vande Broek et al. 1993).

The fact that we could not detect *A. brasilense* or related species associated with roots of the cultivar Pájaro may suggests that for some yet unknown reason this cultivar may not support the plant/bacterium interaction expected for this ubiquitous

Fig. 5 Total indoles production of different *A. brasilense* strains isolated from strawberry plants grown in N-free NFb semisolid medium without (**A**) and with (**B**) Trp supplementation (0.1 mg ml^{-1}). RLC1 to 8: correspond to strains isolated from washed roots; REC1 to 7: strains isolated from surface sterilized roots; PEC1 to 5: strains isolated from inner tissues of first stolons. Sp7: reference strain of *A. brasilense*. Data are the means of three determinations and the *error bars* indicate SD. *Different letters* indicate significant differences at $P = 0.05$
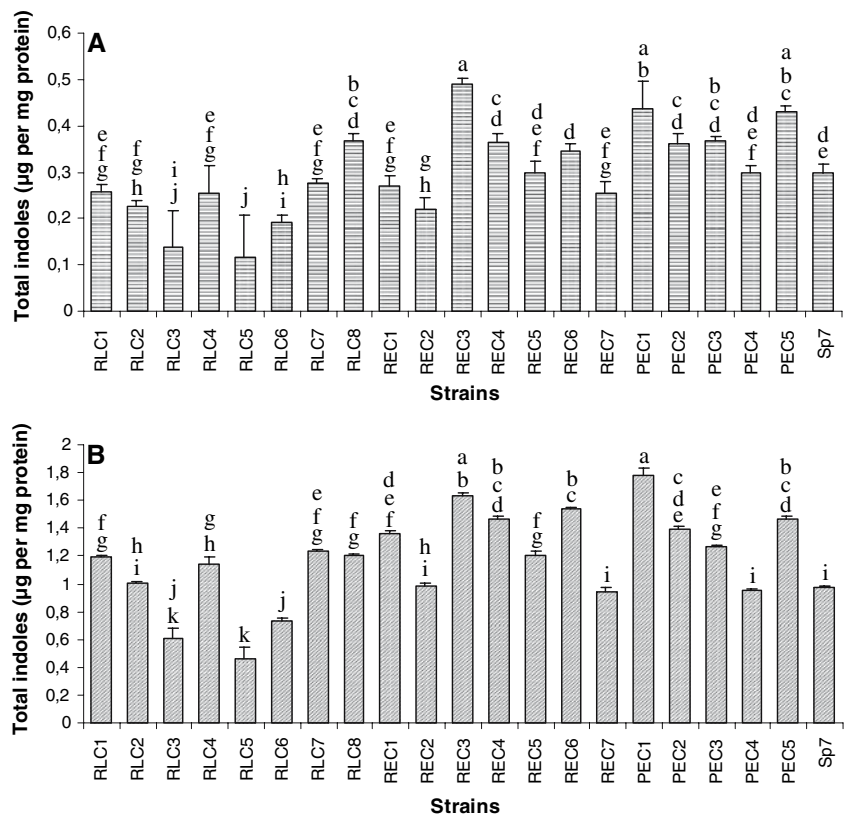
and none-plant-specific genera. We should mention that Camarosa has shown to be better adapted to the soils of Tucumán (Pérez and Mazzone 2004) and according to results obtained in this work, presents associative affinity with *Azospirillum*. This difference could explain, in part, and from a microbiological point of view, the success of the cultivar Camarosa at field (Pérez and Mazzone 2004).

Although all the *Azospirillum* strains were isolated from Camarosa, the MPN showed differences of around one order of magnitude depending on the tissues analyzed. The bacterial density per gram of plant tissue was ∼100 times higher for bacteria isolated from inner tissues of roots than those present in the first stolon; and the fact that we could not isolate *Azospirillum* from the third stolon in the cultivar Camarosa, may be due to the ratio of stolons growth rate versus velocity of bacterial translocation through inner tissues. In this context, we should assume that the stolon growth rate is higher than the bacterial translocation rate, although some earlier information about transportation of *Azospirillum* in growing roots (not stolons) showed that *Azospirillum* translocates faster than wheat growing roots, irrespective of the root growth rate (Bashan and Levanony 1989).

Azospirillum has been isolated worldwide from different plant tissues but mainly from roots (Bashan and Holguin 1997). Only very few publications on aerial colonization of the phylosphere by *Azospirillum* exist, (Bashan 1998; Bahan and de-Bashan 2002). In this work, the natural occurrence of *Azospirillum* in stolons of strawberry plants constitutes a new report on endophytic aerial colonization by this genus. The fact of isolating *Azospirillum* colonizing inner tissues of stolons offers an agronomic advantage, considering the asexual multiplication of these plants in nursery, by fixing the stolons into the soil. If strawberry plants are inoculated with strains of *Azospirillum*, selected by their plant growth-promoting characteristics, the presence of these bacteria will be assured in the descendants.

According to the microbiological and molecular characterization all the local isolates corresponded to *A. brasilense*. The only isolation of *A. brasilense* in strawberry plants confirms data reported by Pedraza and Díaz Ricci (2003) about the prevalence of this species when studying azospirilla associated with sugarcane plants in the province of Tucumán,

although the presence of *A. amazonense* in sugarcane roots was also reported by the same authors in the same province (Pedraza and Díaz Ricci 2000).

The potential N_2-fixing capacity was evaluated by a molecular approach, amplifying by PCR a 710 bp fragment of the gene *nif*D of the nitrogenase. All local *Azospirillum* isolates harbored this gene fragment, being in agreement with results obtained by Potrich et al. (2001). This feature was confirmed by the ARA, showing positive activity in all the local isolates assessed.

Siderophore-type compounds were excreted by all the *Azospirillum* isolates and diffused through the NFb-CAS agar producing a color change from blue to orange. This characteristic could give an added value as PGPB to the local strains assessed here.

Higher production of total indoles (around fivefold) was observed when the growth-culture-medium was supplemented with Trp, being in agreement with previous reports (Baca et al. 1994; Hartmann et al. 1983; Pedraza et al. 2004; Radwan et al. 2002; Reynders and Vlassak 1979). The N-free NFb semisolid medium showed to be suitable for indolic compounds biosynthesis by *Azospirillum*. This could be due to the components of its formula and the microaerobic condition (semisolid), which would stimulate the nitrogenase activity coupled to the enzymatic system involved in auxin biosynthesis. In this regard, higher amounts of indolic compounds were observed by Radwan et al. (2002) when assessing the production in NFb semisolid media than in liquid media. The fact of observing local indoles-producing strains of *Azospirillum* is a relevant feature of PGPB considering that some mutants impaired in indole-3-acetic acid production are not doing well in promoting plant growth (Dobbelaere et al. 1999; Rodríguez et al. 2006).

Our results also suggest, that since the local isolates showed different yields in total indoles production and nitrogenase activity (ARA), the isolates may be described as different strains.

In conclusion, this work constitute the first report of the natural occurrence of *A. brasilense* in strawberry plants. We have obtained different *Azospirillum* strains colonizing the surface and inner tissues of roots as well as inner tissues of stolons, and the latter also correspond to the first report of *Azospirillum* colonizing aerial inner tissues of stolons. All the isolates showed to fix nitrogen, to produce

siderophores, and indoles, three important characteristics within the PGPB group.

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