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## More than rhizosphere colonization of strawberry plants by *Azospirillum brasilense*

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### ARTICLE INFO

#### Article history:

Received 22 June 2011

Received in revised form

13 September 2011

Accepted 21 October 2011

#### Keywords:

*Azospirillum brasilense*

Electron microscopy

*Fragaria ananassa*

nifD

PGPB

Stolon

### ABSTRACT

*Azospirillum brasilense* is a plant growth promoting bacteria (PGPB), capable of improving growth and yield of important crops including strawberry (*Fragaria ananassa*, Duch.). Considering that for commercial purposes strawberry plants are asexually reproduced by planting stolons into the soil, the aim of this work was to evaluate *Azospirillum* root association and further colonization through stolons from bacterial inoculated strawberry mother-plants to new born daughter-plants. For that, three commercial cultivars of strawberry ('Camarosa', 'Milsei' and 'Selva') were root inoculated with two strains of *A. brasilense*: REC3 and PEC5. Scanning and transmission electron microscopy observations showed clear bacterial attachment to the root surface and colonization of root and stolon inner tissues. The diazotrophic bacteria were re-isolated from inoculated mother-plants, non-inoculated daughters and stolons using N-free NFB semisolid medium. In all cases, the MPN from root samples was higher than from stolons. The bacterial *nifD* gene, essential in the biological N<sub>2</sub>-fixing process, was PCR-amplified from DNA of roots and stolons proving the occurrence of diazotrophs within these tissues. To confirm that these bacteria corresponded to the inoculated PGPB, the 16S rDNA gene of re-isolates was subjected to amplified rDNA restriction analysis (ARDRA) and to automated DNA sequencing, revealing that they belong to *Azospirillum brasilense*. This confirms effective rhizosphere colonization of strawberry mother-plants and also the colonization of *A. brasilense* to new daughter-plants via stolons. This is the first report about *A. brasilense* colonization from one strawberry plant to another one by colonizing inner tissues of roots and stolons. This means that a single inoculation with selected PGPB would allow the growers to have numerous plant generations at nursery already inoculated and with better conditions to be planted at field, contributing to a sustainable agricultural practice.

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### 1. 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 (Dobbelaere et al., 2003; Bashan and de-Bashan, 2010). *Azospirillum* are generally referred to as rhizosphere bacteria, displaying strain-specific differences concerning root colonization. They predominantly colonize the root surface; however, some strains are able to colonize plant internal tissues as endophytic bacteria that invade tissues of living plants showing no external signs of infection or negative effects on the host (Steehnoudt and Vanderleyden, 2000; Schulz and Boyle, 2006). Ultrastructural

studies of *Azospirillum* localization on root surface revealed that it can be found all along inoculated root system (Bashan et al., 2004), but they are concentrated on the elongation zone (Bashan et al., 1986; Bashan and Levanony, 1989a; Levanony et al., 1989), root tips (Bashan and Levanony, 1989b), the base of root hairs (Kapulnik et al., 1985) and in few cases on root hair tips (Bashan and Levanony, 1989a).

In a previous work, different *Azospirillum brasilense* strains were isolated from strawberry plants (Pedraza et al., 2007). They showed three important characteristics within the PGPB group: nitrogen fixing activity, production of siderophores and indoles. Also, they were able to promote plant growth of strawberry plants (Pedraza et al., 2010) and protect them against anthracnose disease (Tortora et al., 2011a,b). However, until now, there was not morphological and molecular evidence of strawberry root and stolon colonization by *Azospirillum*.

Strawberry is a worldwide important perennial fruit crop and for commercial purposes it is asexually reproduced at nursery by

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planting stolons into the soil to obtain newborn daughter-plants, without detaching them from the mother-plant. In strawberry plants, a stolon is a branch that emerges from the union of shoot and roots and grows horizontally above the ground producing a new plant at nodes.

Considering root colonization as a key factor in the successful interaction between plants and *Azospirillum*, the achievement of effective strawberry root colonization will ensure the presence of bacteria in the mother-plant root system. Furthermore, this would improve their chances of reaching daughter-plants root system by their colonization through stolons. With this assumption, a single inoculation with *Azospirillum* would allow the growers to have numerous plant generations at nursery already inoculated with selected PGPB and with better conditions to be planted at field. Therefore, the working hypothesis is that *Azospirillum* can colonize strawberry roots of inoculated mother-plants and also colonize non-inoculated daughter plants through stolons. Hence, the aim of this work was to evaluate by microbiological, ultrastructural and molecular methods the root association of *A. brasilense* and its colonization through stolons from inoculated strawberry mother-plants to non-inoculated new born plants.

## 2. Materials and methods

### 2.1. Microorganisms and inoculum preparation

Two strains of *A. brasilense*, REC3 and PEC5, previously isolated and characterized by Pedraza et al. (2007) were used. Both strains were considered in order to analyze their affinities or differences in their colonization patterns in the tested strawberry cultivars as they were isolated from different plant tissues: REC3 was isolated from strawberry inner root tissues and PEC5 from stolon inner tissues (Pedraza et al., 2007).

For inoculation of strawberry roots, pure bacterial cultures of each strain containing about  $10^6$  CFU ml<sup>-1</sup> were prepared in NFB liquid medium as described by Pedraza et al. (2010). Also, a loop-full of each *A. brasilense* pure culture previously grown in NFB semisolid medium (Baldani and Döbereiner, 1980) was streaked out on Petri dishes containing NFB solid medium with NH<sub>4</sub>Cl 1% (w/v). After 120 h at 30 °C samples of agar containing *Azospirillum* colonies were processed to be analyzed by scanning electron microscopy.

### 2.2. Plant material

Three commercial cultivars of strawberry (*Fragaria ananassa*, Duch) were used: 'Camarosa', 'Milsei', and 'Selva'. Healthy and bacteria free plantlets were obtained from *in vitro* culture at the Active Germoplasm Bank at National University of Tucumán. The plantlets were tested for the absence of microbes by plating root and leaf macerates in trypticase soy agar medium (TSA) (Difco-BBL, Sparks, MD) and incubating for 120 h at 30 °C.

### 2.3. Inoculation and hydroponic plant growth conditions

To study *A. brasilense* colonization of strawberry roots at ultrastructural level, plants were grown in hydroponic conditions to avoid soil particles that could disturb visualization of superficial structures. Strawberry vitroplants were aseptically grown on test tubes (50 ml) containing 20 ml 50% Hoagland solution (Hoagland, 1975). Five plants of each cultivar were independently inoculated with 1 ml of bacterial suspension (about  $10^6$  CFU ml<sup>-1</sup>) containing strain REC3 or PEC5. Five non-inoculated plants of each cultivar were considered as controls. The experimental design was completely randomized. Plants were kept in a growth chamber at 28 °C, 70% relative humidity (RH) and 16 h of photoperiod

(250 μmol photons m<sup>2</sup> s<sup>-1</sup>). Root samples were collected 13 days after inoculation and immediately processed for transmission and scanning electron microscopy. Ultrastructural observations were done on three specific root zones: apex, elongation and root hair.

### 2.4. Inoculation and plant growth conditions used to study *Azospirillum* colonization in different plant generations

*Azospirillum* inoculation of strawberry plants was carried out following the experimental design described above but in this case roots were submerged in each *Azospirillum* suspension (about  $10^6$  CFU ml<sup>-1</sup>) for 20 min and then were individually planted in disinfected pots containing sterile substrate (humus-perlome; 2:1). Control plants were submerged 20 min in sterile distilled water.

Plantlets were placed in a growth chamber during 7 months at 28 °C, 70% RH and 16 h of photoperiod (250 μmol photons m<sup>2</sup> s<sup>-1</sup>). During this period, they received 50 ml of sterile distilled water every other day and the growing stolons were planted in different disinfected pots filled with sterile substrate in order to obtain new daughter-plants. After 7 months of growth, samples of roots and stolons of each plant were collected separately to evaluate the presence of *A. brasilense* strains by ultrastructural, microbiological, and molecular methods.

### 2.5. Scanning and transmission electron microscopy (SEM and TEM)

Samples of *Azospirillum* colonies grown on agar-plates, inoculated and non-inoculated roots, and stolons longitudinally sliced were processed for SEM and TEM.

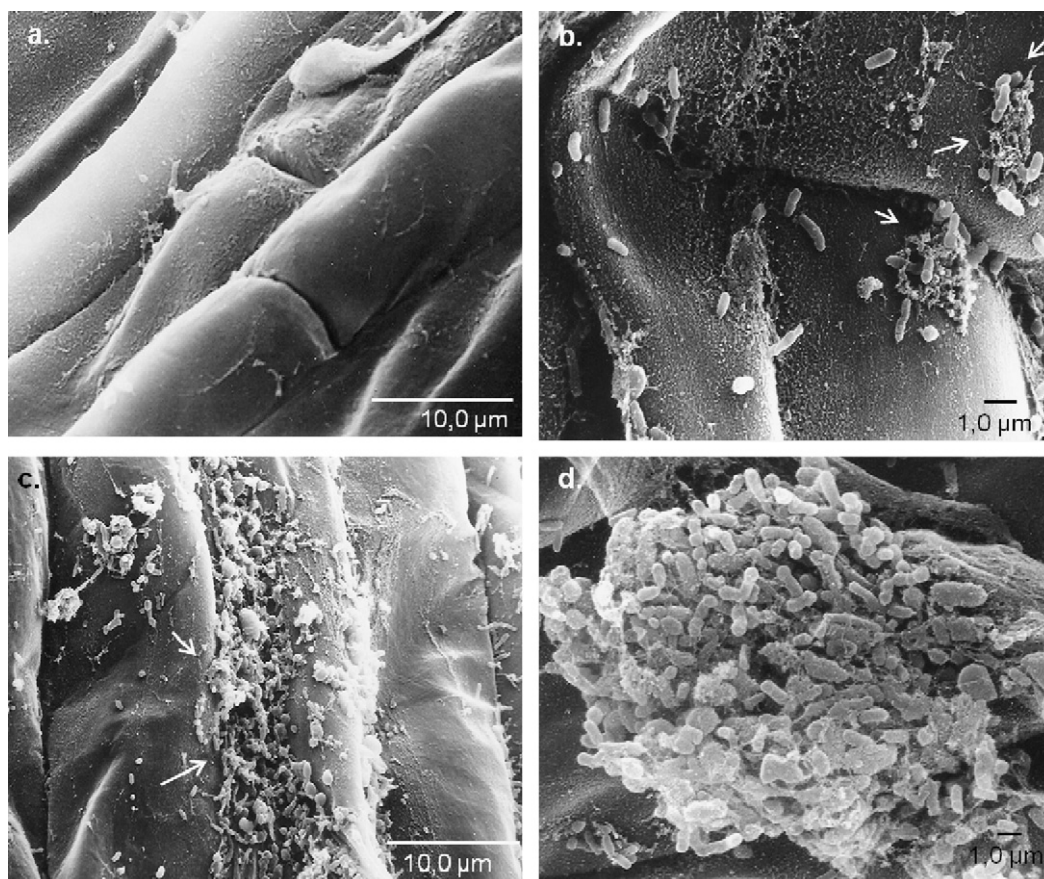
For SEM, samples were fixed in 3% (v/v) glutaraldehyde solution buffered with 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature and postfixed in 1% (v/v) osmium tetroxide in the same buffer. Specimens were washed three times in sterile distilled water and treated with aqueous solution of uranyl acetate 2% (w/v) for 40 min. After fixation, samples were dehydrated through a graded ethanol series (30–100%) followed with acetone (100%), critical point dried, mounted on aluminum stubs, coated with gold and examined with JEOL JSM35 CF (Jeol Co. Ltd., China) and ZEISS SUPRA 55VP (Carl Zeiss Co., Germany) scanning electron microscopes.

TEM samples were identically prepared until the dehydration step, and then they were immediately embedded in Spurr Resin (Spurr, 1969). Ultrathin sections were cut, mounted on cooper grids, stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined with a ZEISS EM 109 (Carl Zeiss Co., Germany) transmission electron microscope.

### 2.6. Isolation and quantification of *Azospirillum*

Isolation and quantification of *Azospirillum* was carried out from serial dilutions of root and stolon macerates in phosphate buffer (pH 7.0). From each dilution, 0.1 ml was inoculated per triplicate in vials containing 5 ml of N-free NFB semisolid medium and incubated for 72 h at 30 °C.

The cultures forming a typical white pellicle below the surface of the semisolid medium were considered positive after checking its morphology and motility by optical microscopy. The occurrence of *A. brasilense* on these cultures was checked by ARDRA and 16S rDNA automated sequencing (Section 2.8). Most probable number (MPN) of *Azospirillum* per fresh gram of root or stolon was estimated in triplicates in NFB semisolid medium using the McCrady probability table for three replicates according to Pedraza et al. (2007).



**Fig. 1.** Scanning electron micrographs of strawberry roots colonized by *A. brasilense*. (a) Control roots without associated bacteria; (b) bacteria as single cells on the root surface; (c) bacterial aggregates; (d) biofilm formation. Arrows indicate zones with granular-like material.

Values were expressed as number of bacterial cells per gram of fresh vegetal weight and results were subjected to ANOVA and LSD ( $p \leq 0.05$ ) analysis with the Statistix Analytical Software 1996 for Windows.

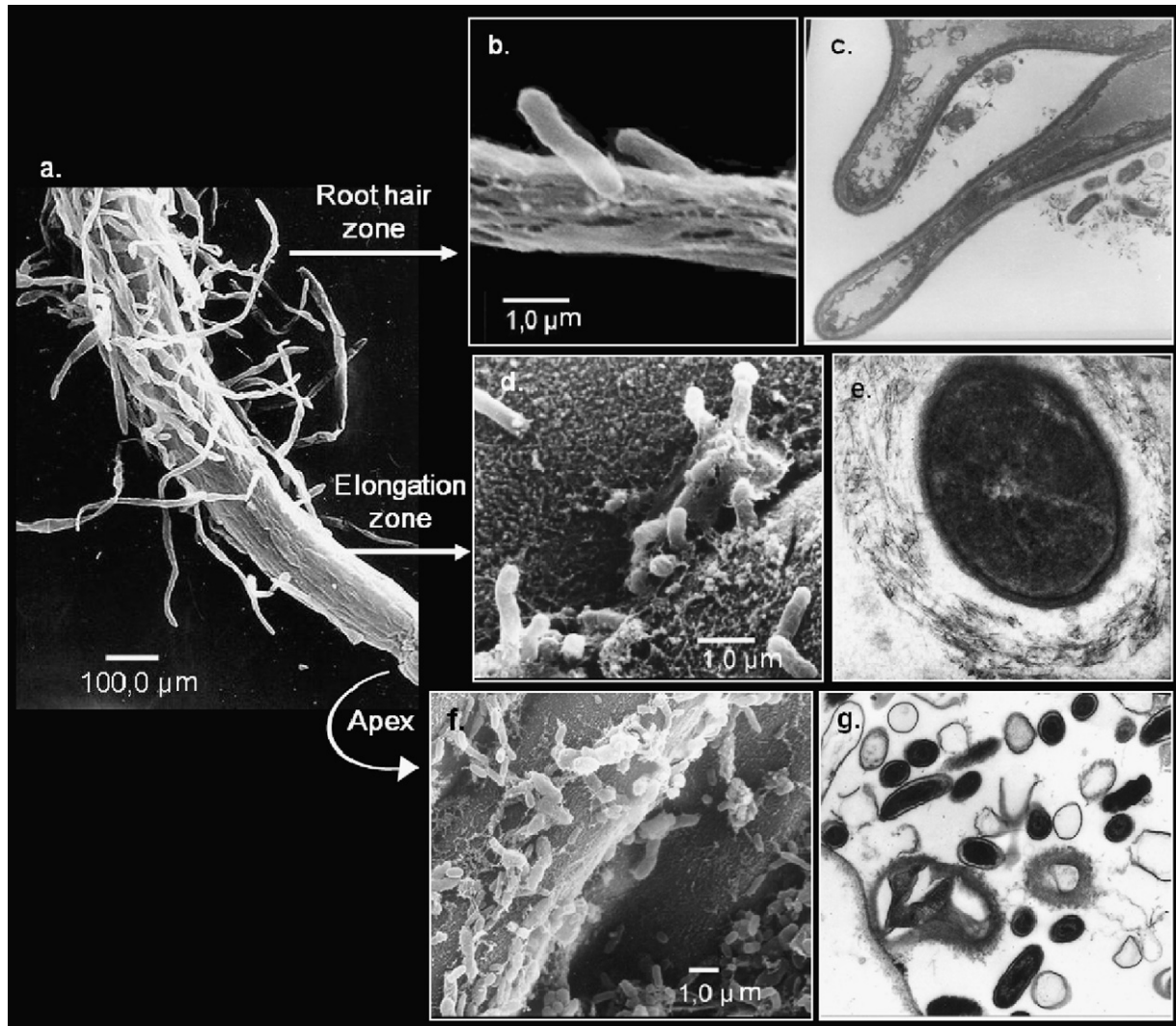
### 2.7. PCR-amplification of *nifD* gene

The occurrence of diazotrophic bacteria within plant tissues was assessed by PCR-amplification of a 710 bp fragment of *nifD* gene (nitrogenase enzyme) using specific primers: *nifD*-up (5'ATCATCGGTGACTACAAC) and *nifD*-do (5'ATCCATGTCGCGGCGAA) described by Potrich et al. (2001). The template DNA for *nifD*-PCR was obtained by the CTAB method (Doyle and Doyle, 1987) from roots and stolons of inoculated strawberry mother-plants, their daughter-plants and non-inoculated control plants. The PCR mixture contained 1× Green GoTaq Reaction Buffer (Promega; pH 8.5);  $MgCl_2$  1.5 mmol $l^{-1}$ ; dNTPs 400  $\mu$ mol $l^{-1}$ ; 0.4  $\mu$ mol $l^{-1}$  of each primer; 2U of *Taq* DNA polymerase (Promega); 0.2  $\mu$ g of template DNA and distilled water to 25  $\mu$ l of final volume. DNA of *A. brasilense* Sp7 (ATCC 29145) was used as positive control. PCR was performed using an Apollo ATC-201 thermocycler (Continental Lab Products, San Diego, CA, USA) with the following conditions: 95 °C for 3 min; 35 cycles of denaturation at 95 °C for 1 min, annealing 52 °C for 1 min and extension at 72 °C for 2 min. PCR products were analyzed by agarose gel (1.0%, w/v) electrophoresis at 5.0 V/cm and revealed by ethidium bromide staining (Sambrook et al., 1989). Digital images were acquired by using the 1D Image Analysis Software (Kodak Digital Science™) with digital camera Kodak Z712IS.

### 2.8. 16S rDNA-amplification, ARDRA and 16S rDNA sequencing

The identification of *A. brasilense* in the bacterial re-isolates obtained from root and stolon tissues was assessed by amplified 16S rDNA restriction analysis (ARDRA) and 16S rDNA gene automated sequencing. For that purpose, 16S rDNA-amplification was performed directly on single bacterial colonies grown on NFB solid medium. For each PCR reaction, a whole bacterial colony was picked from agar plate, suspended in 20  $\mu$ l of sterile distilled water and heated 10 min at 95 °C to allow cell lysis; 1  $\mu$ l of the lysed cell suspension was used as template. The amplification of a 1450 bp fragment of the 16S rDNA gene was carried out using the primers 27f (5'-GAGAGTTTGATCCTGGCTCAG) and 149r (5'-CTACGGCTACCTTGTACGA) (annealing temperature: 60 °C) (Grifoni et al., 1995). DNA of *A. brasilense* REC3, PEC5 and Sp7 (ATCC 29145) were used as positive control. Positive 16S rDNA-amplicons (15  $\mu$ l) were digested with 5 U of *AluI* (Promega) in a final volume of 30  $\mu$ l at 37 °C for 3 h. The restricted fragments were separated by agarose gel (1.5%, w/v) electrophoresis using TBE 1× buffer to obtain ARDRA profiles.

DNA sequencing of 16S rDNA gene of four representative isolates (bacteria isolated from mother plant, 1st, 2nd and 3rd daughter plants) was performed by the dideoxy chain termination method (Sanger et al., 1977). Sequencing reaction was carried out using 4-capillary ABI 3130/Hitachi Genetic Analyzer (Applied Biosystems, USA) and the oligonucleotides 27f and 149r, described above. Sequence analysis was performed with nucleotide sequences available in the GenBank, including *A. brasilense* REC3 (FJ012319.1), EMBL, DDBJ and PDB databases using BLASTN 2.2.25 program



**Fig. 2.** Scanning and transmission electron micrographs of three different zones of a strawberry root colonized by *Azospirillum*. (a) Root inoculated with *A. brasilense*; (b, c) root hair zone; (d, e) elongation zone; (f, g) root apex. Note the fibrillar-like material on top of bacterial cells.

through the National Center for Biotechnology Information (NCBI) server (Zheng et al., 2000).

### 3. Results

#### 3.1. Ultrastructural results

SEM observations of all inoculated plants from hydroponic experiments showed different degrees of bacterial colonization on the root surface: areas with low density of associated bacteria randomly dispersed, zones with bacterial aggregates, and other areas with biofilms covering the root surface (Fig. 1b–d). Most of these bacterial cells had a non-polar orientation, being parallel to the root surface. In contrast, no bacterium was observed on the surface of control plants (Fig. 1a).

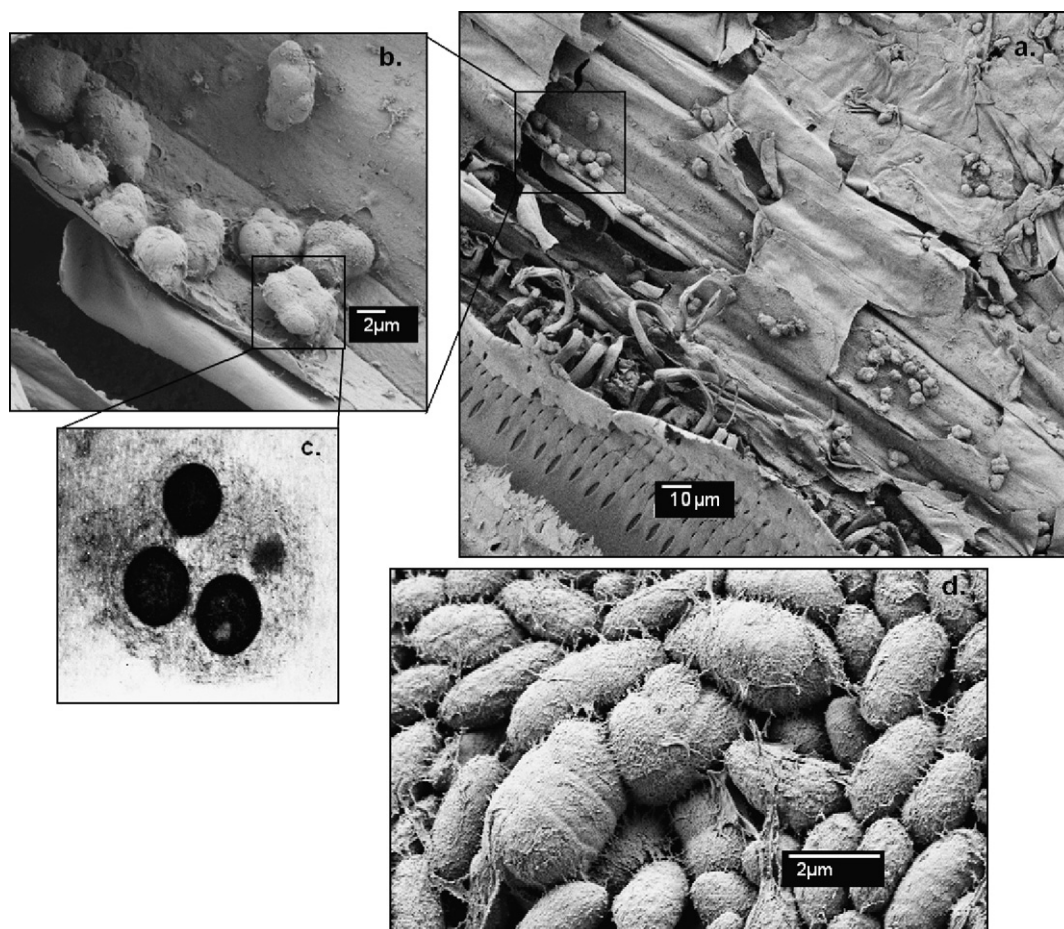
To study the location of *A. brasilense* along strawberry roots, three root zones (apex, elongation and root hairs) were observed (Fig. 2a). SEM of inoculated roots showed massive bacterial colonization on the elongation zone and root apex (Fig. 2d, f), whereas only few bacteria were observed on root hairs (Fig. 2b, c).

Similarly to SEM observations, transmission electron micrographs of root apex confirmed that this area was extensively colonized by bacteria (Fig. 2g). In addition, TEM showed that

inside root tissues, bacteria were surrounded by an electron-dense filamentous material disposed in a concentric arrangement. The presence of an electron-lucid area between the surrounding filamentous material and the bacterial outer cell membranes revealed that there are not binding structures among them (Fig. 2e).

Bacteria were also found attached to root epidermal cells by a fibrillar-like material (Fig. 2f). This latter was probably produced by the bacteria growing on the roots, as it was also observed over bacterial cells forming a single colony on agar-plate (Fig. 3d). Inoculated roots showed granular-like material as discrete solid particles accumulated on the root surface; and zones with high concentration of that material also showed a large amount of bacteria attached to the root (Fig. 1b, c).

Our first evidence that *Azospirillum* colonizes stolons was inferred by the observation of bacteria inside uninoculated daughter-plant roots examined by TEM, as compared to control roots without associated bacteria. However, the most certain evidences of stolon colonization by *A. brasilense* emerged from SEM and TEM observations of stolon inner tissues, which revealed bacterial cells gathered in groups and enclosed with a condensed capsular material in the vascular vessels area (Fig. 3a–c). Similar ultrastructural characteristics were observed in pure colonies



**Fig. 3.** Stolon inner structures of strawberry plants showing associated bacteria resulting from the root inoculation with *A. brasilense* PEC5. (a) Bacteria gathered on groups in the stolon vascular zone; (b) magnification of (a); (c) transmission electron micrograph of bacteria immersed in a matrix formed by a material with concentrically disposed (18,700 $\times$ ); (d) scanning electron micrograph of a pure colony of *A. brasilense* PEC5 grown on NFB solid medium (note the fibrillar-like material produced by bacterial cells).

grown on NFB solid medium (Fig. 3d). Additionally, *A. brasilense* was isolated and quantified from roots and stolons of all inoculated mother-plants and their uninoculated daughter-plants, confirming the ultrastructural results.

### 3.2. MPN determinations

Quantification of *Azospirillum* is shown in Table 1. In all cases, the MPN of *Azospirillum* per fresh-vegetal-weight was higher for roots than for stolons ( $P=0.05$ ). Values ranged from  $1.5 \pm 0.5 \times 10^3$  to  $4.5 \pm 0.5 \times 10^6$  bacteria  $g^{-1}$  of fresh root and from  $1.1 \pm 0.4 \times 10^1$  to  $4.5 \pm 0.5 \times 10^4$  bacteria  $g^{-1}$  of fresh stolon. Statistical analysis showed that the MPN of roots were higher for mother plants and decreased towards newer tissues (mother > 1st > 2nd > 3rd daughter). Conversely, values of MPN in the 3rd and 4th stolon were significantly higher than the 1st and 2nd stolon (Table 1). Furthermore, the ANOVA and post hoc LSD test of strains REC3 and PEC5 showed significant differences in their affinity for each strawberry cultivar as it was previously pointed out by Pedraza et al. (2010). The MPN of cv 'Camarosa' and 'Milsei' were higher when inoculated with REC3 than PEC5, while for cv 'Selva' was the opposite.

*In vitro* strawberry plantlets were tested for the presence of microbes before inoculation with *A. brasilense*. It was not detected any microbe; neither isolated any bacterium from the macerates of these plantlets cultured in TSA and NFB media, similar to that observed for roots and stolons macerates of non-inoculated controls plants (Table 1).

### 3.3. Molecular analysis

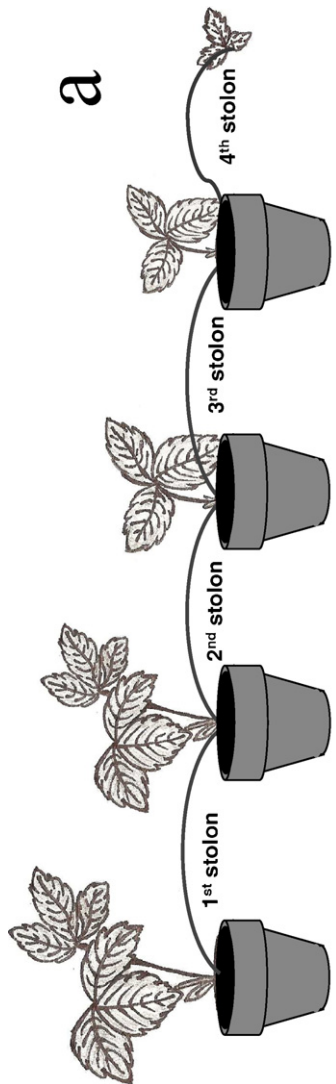
To verify the presence of diazotrophic bacteria within strawberry plant tissues a 710 bp fragment of the *nifD* gene was amplified by PCR from DNA samples obtained from root (Fig. 4c) and stolon tissues (Fig. 4d) colonized by *Azospirillum*. Fig. 4 shows the PCR-products of some representative samples from inoculated plants and positive control strain (*A. brasilense* Sp7), whereas no band was observed in samples from control plants (not inoculated with *A. brasilense*).

The identification of strains REC3 and PEC5 (inoculated and re-isolated) was based on molecular tests specifically reported for *Azospirillum* species (Grifoni et al., 1995). The 16S rDNA gene was PCR-amplified obtaining a specific band of 1450 bp from DNA samples of bacteria isolated from mother- and daughter-plants colonized by *Azospirillum* (Fig. 4a). The restriction profiles of 16S rDNA gene digested with the endonuclease *AluI* confirmed that the bacterial strains re-isolated from strawberry tissues correspond to *A. brasilense*, in comparison with the strains REC3, PEC5 and Sp7 of *A. brasilense* used as positive controls (Fig. 4b). Additionally, sequence analysis of partial 16S rDNA sequences (500 bp) revealed that the tested re-isolates belong to *Azospirillum brasilense* with a  $\geq 99\%$  similarity score.

## 4. Discussion

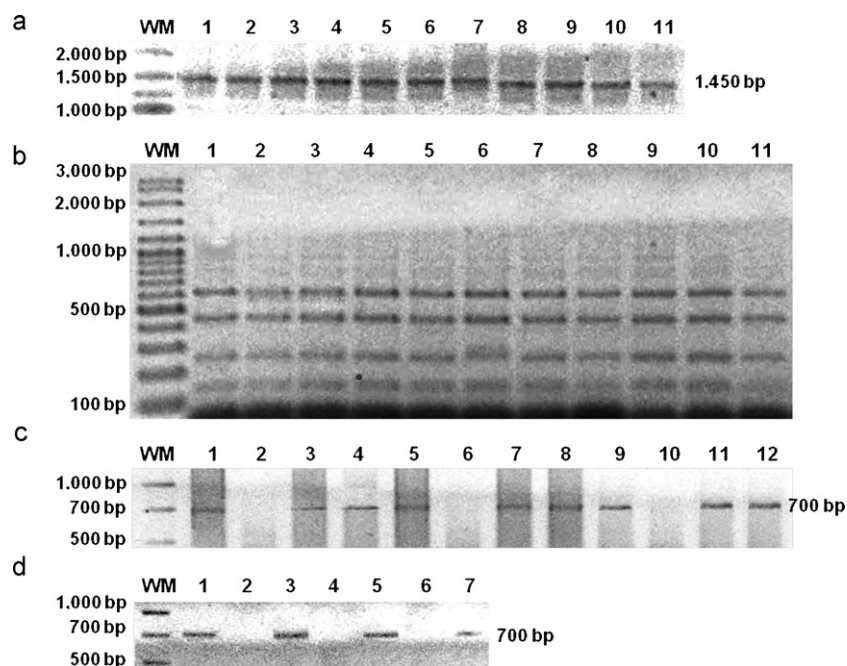
The anchoring of *A. brasilense* to the root surface by a network of fibrillar-like material and their different adsorption patterns

**Table 1**  
Enumeration of *A. brasiliense* strains in strawberry roots and stolons using the MPN method.



Roots and stolons samples Treatments	Mother plant (inoculated with <i>A. brasiliense</i> )		1 <sup>st</sup> daughter plant		2 <sup>nd</sup> daughter plant		3 <sup>rd</sup> daughter plant		4 <sup>th</sup> daughter plant	
	1 <sup>st</sup> Stolon	1 <sup>st</sup> Daughter root	2 <sup>nd</sup> Stolon	2 <sup>nd</sup> Daughter root	3 <sup>rd</sup> Stolon	3 <sup>rd</sup> Daughter root	4 <sup>th</sup> Stolon	4 <sup>th</sup> Daughter root	5 <sup>th</sup> Stolon	5 <sup>th</sup> Daughter root
Camarosa-Control	0	0	0	0	na	na	na	na	na	na
Camarosa-REC3	$4.5 \pm 0.5 \times 10^6$ a	$1.5 \pm 0.4 \times 10^2$ b	$1.5 \pm 0.4 \times 10^2$ b	$9.7 \pm 0.8 \times 10^4$ ab	$3.1 \pm 0.4 \times 10^3$ b	$3.0 \pm 0.5 \times 10^4$ ab	$7.4 \pm 0.6 \times 10^1$ a	$1.9 \pm 0.4 \times 10^4$ c	$2.5 \pm 0.5 \times 10^2$ a	$2.5 \pm 0.5 \times 10^2$ a
Camarosa-PEC5	$1.4 \pm 0.3 \times 10^4$ a	$1.5 \pm 0.4 \times 10^2$ b	$1.5 \pm 0.4 \times 10^2$ b	$9.5 \pm 0.5 \times 10^4$ ab	$1.5 \pm 0.5 \times 10^2$ b	$4.5 \pm 0.5 \times 10^4$ ab	na	na	na	na
Milisei-Control	0	0	0	0	0	0	na	na	na	na
Milisei-REC3	$2.0 \pm 0.4 \times 10^4$ a	$9.6 \pm 0.7 \times 10^1$ b	$9.6 \pm 0.7 \times 10^1$ b	$9.5 \pm 0.6 \times 10^5$ ab	$2.5 \pm 0.5 \times 10^2$ b	$1.6 \pm 0.2 \times 10^5$ ab	$9.7 \pm 0.6 \times 10^1$ a	$3.0 \pm 0.5 \times 10^4$ c	$2.5 \pm 0.5 \times 10^2$ a	$2.5 \pm 0.5 \times 10^2$ a
Milisei-PEC5	$4.5 \pm 0.5 \times 10^3$ a	$1.1 \pm 0.4 \times 10^1$ b	$1.1 \pm 0.4 \times 10^1$ b	$9.7 \pm 0.8 \times 10^3$ ab	$4.5 \pm 0.3 \times 10^1$ b	$1.5 \pm 0.5 \times 10^3$ ab	na	na	na	na
Selva-Control	0	0	0	0	0	0	0	na	na	na
Selva-REC3	$3.0 \pm 0.4 \times 10^4$ a	$2.5 \pm 0.5 \times 10^1$ b	$2.5 \pm 0.5 \times 10^1$ b	$3.0 \pm 0.5 \times 10^4$ ab	$9.4 \pm 0.4 \times 10^1$ b	$9.5 \pm 0.5 \times 10^5$ ab	$1.5 \pm 0.4 \times 10^2$ a	$2.5 \pm 0.5 \times 10^5$ c	$2.5 \pm 0.5 \times 10^2$ a	$2.5 \pm 0.5 \times 10^2$ a
Selva-PEC5	$3.0 \pm 0.5 \times 10^4$ a	$4.5 \pm 0.5 \times 10^2$ b	$4.5 \pm 0.5 \times 10^2$ b	$1.2 \pm 0.6 \times 10^6$ ab	$4.5 \pm 0.5 \times 10^3$ b	$1.6 \pm 0.4 \times 10^6$ ab	$4.5 \pm 0.5 \times 10^4$ a	$4.6 \pm 0.4 \times 10^5$ c	$4.6 \pm 0.4 \times 10^5$ c	$4.6 \pm 0.4 \times 10^5$ c

<sup>a</sup> Scheme of the vegetative reproduction of strawberry plants; the mother and daughter plants are connected through stolons. Note that only the mother-plant has been inoculated with *A. brasiliense*. Values correspond to the means of three determinations and the standard deviation. Different letters indicate significant differences at  $P=0.05$ . na, not analyzed because the number of root and/or stolon indicated was not produced as a new plant generation.



**Fig. 4.** (a) Electrophoretic patterns of amplified 16S rDNA. Lanes 1–3: *A. brasilense* REC3, PEC5 and Sp7 (positive controls). Lanes 4–11: strains re-isolated from strawberry roots and stolons: 4, mother-plant 'Camarosa'-REC3; 5, 1st daughter 'Camarosa'-REC3; 6, 1st stolon 'Camarosa'-REC3; 7, mother-plant 'Milsei'-REC3; 8, 1st daughter 'Milsei'-PEC5; 9, 1st stolon 'Milsei'-REC3; 10, 2nd daughter 'Selva'-PEC5; 11, 1st stolon 'Selva'-PEC5; WM, molecular weight marker (100 bp DNA Ladder; Genbiotech). (b) 16S rDNA digested with *AluI*. Lanes 1–11 and WM: as previously indicated. (c) Amplified 710 bp *nifD* fragments from DNA obtained from strawberry roots: 1, Positive control *A. brasilense* Sp7; 2, mother-plant 'Camarosa'-control; 3, mother-plant 'Camarosa'-REC3; 4, 1st daughter 'Camarosa'-REC3; 5, 2nd daughter 'Camarosa'-REC3; 6, mother-plant 'Milsei'-control; 7, mother-plant 'Milsei'-REC3; 8, 1st daughter 'Milsei'-REC3; 9, 1st daughter 'Milsei'-PEC5; 10, mother-plant 'Selva'-control; 11, mother-plant 'Selva'-PEC5; 12, 1st daughter 'Selva'-PEC5; WM, molecular weight marker (wide range DNA marker 50–10,000 bp; Sigma). (d) Amplified 710 bp *nifD* fragments from DNA of strawberry stolons: 1, positive control *A. brasilense* Sp7; 2, 1st stolon 'Camarosa'-Control; 3, 1st stolon 'Camarosa'-REC3; 4, 1st stolon 'Milsei'-Control; 5, 1st stolon 'Milsei'-REC3; 6, 1st stolon 'Selva'-Control; 7, 1st stolon 'Selva'-PEC5; WM, molecular weight marker (wide range DNA marker 50–10,000 bp; Sigma).

(randomly dispersed single-cells, non polar orientation, bacterial aggregates, and biofilms) observed at ultrastructural level, are probably common features of this genus as they have been also previously described (Levanony and Bashan, 1991; Levanony et al., 1989; Bashan et al., 1986; Umali-García et al., 1980). As it was demonstrated in this work, *A. brasilense* is able to produce biofilms, an adherent and dynamic microbial population on the surface of strawberry roots. It is known that a high population density provides the opportunity to perform certain processes that single cells cannot accomplish efficiently, such as the production of excreted metabolites or exoenzymes that are only effective above a threshold concentration (Danhorn and Fuqua, 2007).

It was also observed fibrillar-like material over bacteria attached to strawberry roots, probably produced by bacterial cells rather than by plant cells. This fact was supported by SEM observations of *Azospirillum* pure colony cells, which exhibited similar fibrillar-like material on their surface. Vanbleu and Vanderleyden (2003) stated that fibrillar network allows the bacteria to anchor to roots for better access to plant exudates, and reciprocally, allows the plant to reach substances excreted by the bacteria before consumption by other bacteria. Additionally, it provides resistance against external physical forces applied to the root, such as washing and agitation (Bashan et al., 1991). The fibrillar connections observed herein were similar to those exhibited by *A. brasilense* Cd adsorbed to wheat roots (Bashan et al., 1986), grass roots (Umali-García et al., 1980) and sand particles (Bashan et al., 1991). Granular-like material over strawberry root surfaces was also observed, and root zones having this material were mostly colonized by bacteria. The nature of this granular-like material was not elucidated in this work, but it would probably correspond to rhizodepositions participating in the bacteria–plant association.

The high adsorption of bacteria to the elongation zone and apex of strawberry roots agrees with the observations made by Bashan

and Levanony (1989a) for wheat roots. The main colonization of the root surface at the elongation zone and apex might be explained by considering that such growing regions are constantly exuding chemical compounds, which can attract *Azospirillum* cells and provide nutrients for this metabolically active association, as indicated by Bashan et al. (1986).

Also, TEM observations of the inoculated strawberry root apex showed the inner tissues densely populated with the essayed bacteria. This differs from that reported by Levanony et al. (1989), who affirm that wheat root apex inoculated with *A. brasilense* Cd did not contain bacteria in its internal structures. The endophytic behaviour of the strain REC3 was verified in a previous work by using this strain tagged with the green fluorescent protein (*gfp*) gene, colonizing inner root tissues of cv 'Camarosa' and 'Selva' (Guerrero-Molina et al., 2010). Due to the instability of this construction (*A. brasilense* REC3::*gfp*) for periods longer than 2 weeks, it was not included in this study as it lasted 7 months.

Results of bacterial quantification showed large differences in the amounts of *Azospirillum* isolated from roots and stolons of strawberry. Similarly, in a recent study Rodrigues et al. (2008) found larger populations of *A. amazonense* on washed and surface sterilized roots of rice (*Oryza sativa* L.) rather than on aerial plant tissues. The preferential colonization of root tissues confirms that this is the most favourable ecological niche for establishment and development of this genus. The root area is filled with nutrients and has adequate microaerobic conditions for biological nitrogen fixation (Steehnoudt and Vanderleyden, 2000). Conversely, the low stolon colonization (expressed as low MPN and few associated bacteria observed in their inner structures) and the condensed capsular material observed in bacterial cells inside the stolon tissues may reflect an ecological adaptation of these bacteria to an unfavourable environment. Considering that *nif* genes expression is strictly regulated by intracellular oxygen and ammonium



concentrations (Elmerich et al., 1997), the named capsular structure may perhaps works as a protecting shield of bacteria from starvation conditions and oxygen concentration inside the stolons, thus, prolonging their survival period in stolons until they reach a new root. Hence, we infer that inside strawberry plants *A. brasilense* could be using stolons only as a path in its way to colonize the roots of a new born daughter-plant. Moreover, the increasing counts from the first to the last stolon observed herein for cv 'Selva', indicates that the colonization occurs easily on the growing tissues with active metabolism; thus, bacterial colonization may follows the stolons apical growth with high meristematic activity. This is in agreement with Sturz et al. (1999), who affirmed that bacterial endophytes may not only be host specific, but also plant tissue sensitive, reacting and adapting at certain tissue types within the host plant as it develops.

The PCR-detection of bacterial genes *nifD* from plant DNA samples, confirmed the presence of diazotrophic bacteria within colonized-plants (inoculated mother- and non-inoculated daughter-plants). While, the ARDRA analysis and the 16S rDNA gene sequencing gives evidence that the bacteria colonizing strawberry tissues belong to the specie *A. brasilense*. In this work, we have only detected the presence of *nifD* gene inside root and stolon of strawberry plants, but other authors have also proven the expression of *nif* genes of *Azoarcus* BH72 within rice roots, indicating that the bacteria is metabolically active inside plant tissues (Miche et al., 2006).

Concluding, our results showed effective *A. brasilense* root association and further colonization through stolons from single bacterial inoculated strawberry mother-plants to new born daughter-plants. Taking into account that strawberry plants are commercially reproduced at nursery by planting stolons into the soil, from our findings arise the possibility of using this biotechnological approach to obtain numerous new born-plants already colonized with selected PGPB and in better conditions to be planted at field, contributing with a sustainable agricultural practice.

## Acknowledgements

This work was partially supported by Consejo de Investigación, Universidad Nacional de Tucumán (CIUNT) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 2007 N° 472). We thank Gustavo Martínez-Zamora for technical advices in the sequence analysis. MFGM is CONICET fellow.

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