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REGULAR ARTICLE

Endophytic colonization of sugarcane (*Saccharum officinarum*) by the novel diazotrophs *Shinella* sp. UYSO24 and *Enterobacter* sp. UYSO10

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Abstract

Aims Sugarcane is a multipurpose crop mostly used in Uruguay for bioethanol production. It requires high amounts of N fertilization for optimal growth, which causes environmental degradation and high production costs. Previously, a bacterial collection associated with surface-sterilized stems of sugarcane was characterized for in vitro plant growth-promoting (PGP) traits. The aims of this study were (1) to determine if selected isolates from the collection are sugarcane growth promoters and (2) to determine if they are true endophytes of sugarcane.

Methods Plant growth promotion assays were used to study the effects of selected isolates on sugarcane plantlets. Light microscopy, transmission electron, and scanning electron microscopy (TEM, SEM) were employed to describe the structure of the interaction between the plant growth-promoting bacteria and the plants. qPCR was used to quantify the bacteria residing in the inner plant tissues.

Results *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 were confirmed to have a PGP effect on the commercial sugarcane cv. LCP 85384. Both strains were defined as true endophytes of sugarcane plants with this being the first case for a strain in the genus *Shinella* in grasses.

Conclusions These data will contribute to the final development of a sugarcane PGP inoculant based on endophytic plant growth-promoting bacteria.

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Introduction

The Uruguayan agro-industrial sector has been strongly stimulated due to the promotion of biofuel production using renewable, nationally produced feedstocks,

particularly sugarcane (*Saccharum officinarum*), which is the main feedstock employed for bioethanol production. However, this multipurpose crop needs high N fertilizer inputs for optimal growth, which results in high economic and environmental production costs. In Brazil, major efforts have been made to improve the sustainability of sugarcane crops (Boddey et al. 1995; Baldani and Baldani 2005; Reis et al. 2007). Several studies using long-term N balances, ^{15}N natural abundance and ^{15}N isotope dilution methods have shown that some sugarcane cultivars can obtain significant amount of their N requirements through biological nitrogen fixation (BNF) (Urquiaga et al. 1992, 2012), but as yet the bacteria responsible for the BNF measured *in planta* remains unknown (Boddey et al. 1995; James and Olivares 1998; James 2000).

Plant associated bacteria can be classified as rhizospheric when they live in the area influenced by root exudates, epiphytic when colonizing plant surfaces, or endophytic when colonizing the inner tissues of the plant (Hardoim et al. 2008). In particular, a "true endophyte" is defined as a bacterium which has been isolated from surface-sterilized plant tissue and which has microscopic evidence of its presence in the inner tissues (Reinhold-Hurek and Hurek 1998). Endophytic bacteria can directly benefit plant growth by improving the germination rate and nutrient uptake (e.g., of N, P, Fe) as well as by modulating the plant hormone levels and alleviating plant abiotic stresses. Furthermore, indirect benefits include the biological control of phytopathogens as well as the stimulation of systemic-induced resistance in plants (Rosenblueth and Martínez-Romero 2006; Ryan et al. 2008; Mei and Flinn 2010). Considering their ecological niche, it has been suggested that bacterial endophytes may have an ecological advantage over rhizospheric and epiphytic bacteria since they interact more closely with the host, with less competition for carbon sources within a more protected environment (James 2000).

Although the specific microorganisms responsible for the BNF are unknown (James 2000), several diazotrophic bacteria were isolated from the rhizosphere and inner tissues of sugarcane roots and/or stems (Asis et al. 2000; Mirza et al. 2001; Reis et al. 2007; Taulé et al. 2012), and many more have been identified using non-culturable methods (Burbano et al. 2011; Fischer et al. 2012; Thaweenut et al. 2011). For some of the isolated strains it has been demonstrated that their use in inoculation trials, both singly and in consortia,

promotes sugarcane growth (Oliveira et al. 2006, 2009; Taghavi et al. 2009; da Silva et al. 2012). For example, in the specific case of *Gluconacetobacter diazotrophicus*, when it was inoculated onto micropropagated sugarcane plants it promoted sugarcane growth partly by BNF, but also via another mechanism, most likely plant hormones (Sevilla et al. 2001). It is also well known, however, that the effect of bacterial inoculation is highly dependent on the plant genotype, on soil characteristic, and on many other biotic and abiotic factors (Reis Junior et al. 2000; Govindarajan et al. 2006; Carvalho et al. 2011; da Silva et al. 2012).

Rhizobacteria capable of endophytic colonization express particular genes that are required for attachment, penetration, and colonization of the inner plant tissues, allowing their growth and survival within them (Monteiro et al. 2008). Since the molecular basis of plant-endophytic bacterial interactions are not well understood (Turner et al. 2013), additional studies using new models are needed.

In order to contribute to the environmental and economic sustainability of the sugarcane production system in Uruguay, a collection of bacteria previously isolated from surface-sterilized stems, with an emphasis on diazotrophic bacteria, was characterized for in vitro plant growth-promoting (PGP) features (Taulé et al. 2012). In order to gain a better understanding of their interaction with their host plants, and hence assist in their use and management as a potential bioinoculant for sugarcane crops, the aims of this study were to (1) determine by plant inoculation assays if various native diazotrophic bacterial strains are sugarcane growth promoters and (2) determine if they are true endophytes by quantifying and characterizing their colonization of the root surface vis a vis their internal tissue colonization via quantitative real-time PCR (qPCR) and high resolution microscopy.

Materials and methods

Bacterial strains

Bacterial diazotrophs associated with commercial sugarcane cultivars grown in Uruguay were selected from a collection of putatively endophytic strains according to their in vitro PGP features and their phylogenetic relationships (Taulé et al. 2012). The strains studied were *Achromobacter* sp. UYSO02, *Acinetobacter* sp.

UYSO03, *Enterobacter* sp. UYSO10 (proposed *Kosakonia*) (Brady et al. 2013), *Pantoea* sp. UYSO13, *Pseudomonas* sp. UYSO14, *Rahnella* sp. UYSO22, *Shinella* sp. UYSO24, and *Stenotrophomonas* sp. UYSO27. In addition, the well-studied, sugarcane-associated endophyte *G. diazotrophicus* Pal5 (James et al. 2001) was used as a reference strain in the PGP and microscopy experiments.

Screening the selected inoculant strains for potential plant interaction and infection traits

Endoglucanase and hemicellulase activities were screened in solid LGI culture media (Cavalcante and Dobereiner 1998). For endoglucanase and hemicellulase activities the culture media were supplemented with 0.2 % carboxymethyl cellulose (CMC) or 0.5 % avicel, respectively (Kim et al. 2008). In each case, positive strains were identified by degradation halos around each colony.

Protease activity was evaluated in solid media containing LGI medium supplemented with 5 % skim milk. Strains were considered positive when a translucent halo was observed around the colonies (Martinez-Rosales and Castro-Sowinsky 2011).

For peroxidase activity determination, strains were grown on solid media containing LGI medium supplemented with 250 mg l^{-1} of 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS) and for Mn-peroxidases, with 250 mg l^{-1} ABTS plus 100 mg l^{-1} MnCl$_2$.4H$_2$O (Sack et al. 1997). Strains were considered positive when the colonies turned a dark green or brown color.

For determining laccase activity, solid media containing TY medium supplemented with 0.04 % Remazol brilliant blue or with 0.01 % guaiacol were used (Kiiskinen et al. 2004). Strains were considered positive when the colony turned a blue color or when they turned reddish-brown, respectively.

Biofilm formation was screened in 96-well plates using the crystal violet (CV) method (Peeters et al. 2008). Each well containing TY medium or Murashige Skoog (MS) + exudates was inoculated with $1–2 \times 10^8$ cells of each isolate. "MS + exudates" refers to MS medium (Murashige and Skoog 1962) that was exposed to micropropagated sugarcane root secretions for 72 h. The 96-well plates were incubated for 48 h at 30 °C without agitation, after which the supernatant was removed and the wells stained with a 0.1 % CV solution

for 20 min. The excess CV was removed by washing the plates with phosphate-buffered saline (PBS), and the bound CV was solubilized with 95 % ethanol. The absorbance of the suspension was measured at 570 nm. All the aforementioned determinations were performed in triplicate, or in quintuplicate in the case of biofilm determinations.

Micropropagation of sugarcane plants

Shoot tips of sugarcane cv. LCP 85384 were collected from 3-month-old plants grown in sterilized substrate under greenhouse conditions. The shoot tips (4 cm long) were sterilized for 15 min in a 20 % (*v/v*) sodium hypochlorite solution and then exhaustively rinsed with sterile distilled water. Meristems were excised, placed on a sterile filter paper support and transferred to tubes containing 10 mL of full strength MS liquid culture medium supplemented with Staba vitamins (Staba 1969), plant growth regulators (Ponce 1991), and 3 % sucrose. Growth chamber conditions were as follows: 21 °C temperature and 30 μmol m^{-2} s^{-1} irradiance with 16/8 h day/night. After 1 month, the explants were transferred to the MS solid multiplication medium supplemented with 0.1 mg l^{-1} benzyladenine and a mixture of antioxidants (Garcia et al. 2007). Subcultures were repeated every 3 weeks. The plants were rooted in MS solid medium with 1 g l^{-1} of activated charcoal.

Plant growth promotion of micropropagated sugarcane plants

Prior to bacterial inoculation, 4 to 5 rooted micropropagated plantlets of cv. LCP 85384 with similar morphologies were aseptically transferred to flasks containing 20 ml of modified MS medium (Reis et al. 1999). After 3 days, those flasks that did not have any visible contamination were inoculated with 1×10^7 cells plant^{-1} of each strain to be tested. Additionally, an extra treatment (MIX) was included, in which the inoculum was prepared with a mixture of all strains tested. As a negative control, plants were inoculated with 0.1 ml of 0.9 % NaCl. The experiment was randomized with ten replicates per treatment. At 10 d post-inoculation (dpi), plants were transferred to small pots containing sterile sand to vermiculite (2:1) as substrate and were watered normally with MS medium without N, and eventually with MS containing N. Plants were maintained at a temperature of 30 °C with a photoperiod of 16/8 h

light/dark. At 25 dpi, plants were transferred to pots containing 1.5 kg of sterile sand to vermiculite (2:1) as substrate and maintained in the greenhouse with a photoperiod of 16/8 h day/night. The height and diameter of the stems were determined after 4 months. Roots and aerial parts were dried at 65 °C until constant weight for dry weight determination and the total N content of the aerial parts was determined with the Kjeldahl method at the Animal Nutrition Laboratory of the Faculty of Agronomy-UdelaR.

Microscopical examination of the interaction between micropropagated sugarcane plants and plant growth-promoting bacteria

Micropropagated sugarcane plants cv. LCP 85384 were inoculated with bacteria as described above. As a negative control, plants were inoculated with 0.1 ml of 0.9 % NaCl. Plants were harvested at 1, 4, 6, 12, 24, 48 h and at 6 dpi. Two plants per treatment were examined microscopically; the roots and aerial parts for each plant were analyzed independently. Three additional plants were harvested after 48 h and at 5 dpi for bacterial enumeration.

For microscopy, small pieces (1–2 cm) of roots and stem were fixed overnight (o.n.) at room temperature in a solution of 5 % glutaraldehyde, 4 % formaldehyde in 50 mM sodium phosphate buffer (PB), pH 7.2. The fixed samples were rinsed with PB, dehydrated in an ethanol series (15 to 100 %, 15 min per stage), and then infiltrated in medium grade LR white acrylic resin (SIGMA) (James et al. 1994). Semi and ultrathin sections (1-μm and 60–70-nm thickness, respectively) were obtained using an ultramicrotome (Reichert Ultracut S). Semi-thin sections for light microscopy (LM) were collected on glass slides and were stained with 0.2 % toluidine blue, except the sections that were used for immunogold labeling which were kept unstained. LM samples were analyzed with an Olympus IX81 microscope (Olympus Corporation, Tokyo, Japan). For transmission electron microscopy (TEM), the ultrathin sections were collected on Formvar coated nickel grids, and stained for 20 min in 5 % uranyl acetate and 5 min in 0.2 % lead citrate in 0.01 N NaOH, and then washed several times in distilled water (James et al. 1994, 1997). The grids were examined and photographed with a Zeiss EM-900 TEM (Carl Zeiss AG, Jena,Germany) at 80 KV.

Tissues to be analyzed by scanning electron microscopy (SEM) were first cut by hand into 1–2-cm pieces with a new razor blade. After which, the samples were washed with PB buffer, fixed, and dehydrated as previously described. Lastly, the samples were dried in a Critical Point Dryer (Dentom Vacuum Inc.), mounted on metal stubs, and coated with gold-palladium in a Sputter Coater (DESK II). The samples were examined using either a Zeiss DSM-962 or a Jeol 5900 LV SEM operating at 20 KV.

Production of polyclonal antibodies and immunogold labeling for LM and TEM

Polyclonal antibodies raised in rabbits against *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 were produced in the Biotechnology Laboratory of the Pando Technological Pole (Faculty of Chemistry, UdelaR. Uruguay). Briefly, the bacteria were grown in TY liquid medium to log phase, centrifuged, and washed in phosphate-buffered saline (PBS). The pellets were suspended in 3 % formaldehyde in PBS, incubated for 16 h at 4 °C and finally rinsed in PBS. This suspension was used for rabbit inoculation of rabbits.

The cross-reaction of the antibody against several bacteria isolated from the same Uruguayan sugarcane cultivars, such as *Achromobacter* sp. UYSO02, *Acinetobacter* sp. UYSO03, *Pantoea* sp. UYSO13, *Pseudomonas* sp. UYSO14, *Pseudomonas* sp. UYSO21, *Rhanella* sp. UYSO22 (Taulé et al. 2012), as well as the N$_2$-fixing strains *Sinorhizobium meliloti* 242, *Herbaspirillum seropedicae* Z67, and *G. diazotrophicus* Pal5, were carried out in 96-well microtiter plates using the enzyme-linked immunosorbent assay (ELISA) (James et al. 2001). After this first ELISA, the antibodies were purified by adsorption with the two strains that showed most cross-reaction with the antibodies, and the cross-reaction was re-checked by ELISA. For this, cells from each chosen strain were centrifuged from a well-grown 200 ml culture medium and fixed in 0.5 % formaldehyde in PBS for 2 h at 37 °C. After fixing, the cells were rinsed three times with PBS, suspended in a solution of PBS + 3 % skim milk with an antibody dilution of 1:50 and shaken for 2 h at 4 °C. Finally, the suspension was centrifuged and the supernatant used as a specific antibody working solution.

For immunogold labeling (IGL) in LM, semi-thin sections were collected on BioBond-coated glass slides (Life Science, USA) and incubated for 1 h in IGL buffer

(3 % skimmed milk, 0.5 % Tween 20 in 0.5 M PBS pH 7.4) and then for 1 h with the purified primary antibody 1:10 (either anti-*Enterobacter* sp. UYSO10, anti-*Shinella* sp. UYSO24, or non-immune serum). After washing, the slides were incubated for 1 h in a 1:50 dilution of 5 nm goat anti-rabbit gold (British Biocell International, UK) in IGL buffer. The gold labeling was then observed through light microscopy using a silver enhancement kit (BB Solutions, UK) (James et al. 1994). In the case of IGL for TEM, the protocol employed was the same as previously described, but the secondary antibody used was 15 nm goat anti-rabbit gold (BB Solutions, UK). For each immunogold assay the following controls were performed on serial sections: (i) omission of the primary antibody and (ii) replacement of the primary antibody with pre-immune diluted appropriately (1:50) in IGL buffer without Tween 20.

Bacterial enumeration by qPCR

Bacterial enumeration was performed on three replicates of micropropagated sugarcane plants at 48 h and at 6 dpi. The harvested plants were sonicated for 5 min in PBS, vortexed for 1 min in fresh PBS, and the roots and aerial parts separated with a sterile scalpel. DNA was extracted from 200 and 500 mg of the roots and the aerial parts, respectively, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. CA, USA). The amount of DNA obtained was measured at 260 nm and the quality was checked by analyzing the A_{260}/A_{280} and A_{260}/A_{230} ratio and by 1 % agarose gel electrophoresis.

Specific primers for qPCR were designed based on the 16S ribosomal DNA (rDNA) gene sequences of *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24. A nucleotide alignment of 16S rDNA gene was constructed including both strains, and the closest species using the Greengenes program via the NAST alignment tool (De Santis et al. 2006), in which species-specific regions were screened for the designed primers. The selected primers designed were: UYSO10 For (5′-CCGTGCTGATTGACGTTA-3′), UYSO10 Rev (5′-TCACATCCGACTTGACAGAC-3′) and UYSO24 For (5′-TGACTGTAGTCGGAGAAGAAGC-3′), UYSO24Rev (5′-CAGTATCAAAGGCAGTTCCG-3′), respectively. Primer specificity was analyzed in silico using the NCBI BLAST, RDP Probe match, and ProbeCheck tools, as well as in vitro by qPCR using as templates the DNA from several strains also isolated from Uruguayan sugarcane cultivars (Taulé et al. 2012). Finally, the specificity of the amplicon was confirmed by observing the melting curve, by checking the expected size by 1 % gel agarose electrophoresis (143 and 171 bp for UYSO10 and UYSO24, respectively), and by sequencing of the fragment by MACROGEN Inc. (Korea).

The qPCR reaction was performed using the CFX96 Touch Real-Time PCR (BIORAD) equipment and all measurements were performed using the SybrGreen approach. The PCR mixture was iQ SYBR Green Supermix (BIORAD), 1 μM of each primer and 4 to 25 ng of DNA template, all within a total volume of 25 μl. The PCR conditions were as follows: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s (recording fluorescent data), and 72 °C for 30 s. Product specificity was confirmed by melting curve analysis (65–95 °C, increasing by 0.5 °C for each 5 s per read). The standard curves were made for each primer set using the corresponding pure genomic bacterial DNA (*Enterobacter* sp. UYSO10 or *Shinella* sp. UYSO24). The series were ten-fold dilutions and were performed in triplicate. Data were analyzed by BIORAD CFX Manager 3.1.

The number of *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 cells in each tissue was enumerated by an absolute quantification. The copy number for each treatment was calculated by the extrapolation of the standard curve for each bacterial strain. The copy number of the 16S rDNA gene from the DNA was calculated using the formula below (Kim et al. 2013):

DNA copy

$$= \frac{6.02 \times 10^{23} \left(copy / mol \right) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \left(g / mol / bp \right)}$$

Considering that (i) there is only one copy of these genes in both genomes (Beracochea, personal communication) and (ii) an approximation for genome size was taken from the nearest sequenced bacterial strains.

Statistical analyses

An ANOVA test was performed using InfoStat (2008) and when significant differences were confirmed the means were compared using the Tukey test with a $P < 0.05$.

Results

Plant growth promotion of micropropagated sugarcane cv. LCP 85384

Micropropagated sugarcane plants (cv. LCP 85384) inoculated with *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 had significantly greater stem height and shoot dry weight compared to negative controls (Table 1). In addition, those plants inoculated with *Shinella* sp. UYSO24 and *Acinetobacter* sp. UYSO03 had significantly greater root dry weight. Moreover, plants inoculated with *Enterobacter* sp. UYSO10, *Shinella* sp. UYSO24, *Pantoea* sp. UYSO13, *Rahnella* sp. UYSO22, MIX (including a mixture of all the strains tested in this work) and *G. diazotrophicus* Pal5 had significantly greater stem diameter than the negative control. Although there were no significant differences in the plant N concentration between treatments, the plants inoculated with *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 had significantly higher N accumulation values (Table 1).

Plant-bacterial interaction traits of the putatively endophytic strains used as inoculants

With the goal of further characterizing the strains used as inoculants in PGP assays, the presence of several traits that could be involved in plant-bacterial interactions were evaluated. All of the strains tested showed different enzymatic capabilities (Table 2). The most common trait observed was the presence of endoglucanases (five out of six strains tested). In addition, two out of six strains showed protease activity and two others showed laccase activity. None of the strains had hemicellulose or peroxidase activity under test conditions. With regard to biofilm formation on plates, only *Acinetobacter* sp. UYSO03 and *Pseudomonas* sp. UYSO14 were positive under the TY conditions, but *Shinella* sp. UYSO24 was positive on plates containing MS + exudates.

Localization of inoculated bacteria in micropropagated sugarcane cv. LCP 85384

Light and electron (scanning and transmission) microscopy as well as qPCR were used with the aim of describing the surface and inner sugarcane tissue colonization by the inoculated bacterial strains *Enterobacter* sp.

UYSO10 and *Shinella* sp. UYSO24. For the microscopy, and with the aim of specifically identifying the bacteria on and inside the plants, polyclonal antibodies against *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 were raised, purified, and employed in LM and TEM. The cross-reaction of the antibodies with other bacteria associated with sugarcane was evaluated in an ELISA assay. In addition, the specificity of the antibodies were also corroborated on semi-thin sections of samples of various bacteria, including *Enterobacter* sp. UYSO10, *Shinella* sp. UYSO24, *Rhanella* sp. UYSO22, and *Acinetobacter* sp. UYSO03. Control sections did not have any significant gold labeling (data not shown).

For qPCR, specific primers against the *rrs* gene of *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 were designed. The in silico and in vitro evaluation assays confirmed the primer specificity. The lowest amplification efficiencies obtained for *rrs* amplification were 90.2 % for *Enterobacter* sp. UYSO10, and 91.2 % for *Shinella* sp. UYSO24, with r^2 values of 0.993 and 0.997, and slopes of −3.581 to −3.553, respectively.

Colonization of sugarcane cv. LCP 85384 by *Enterobacter* sp. UYSO10

Single rod-shaped bacteria were detected adhering to the surfaces of the roots and lower stems in a non-polar manner at 6 h after inoculation (Fig. 1a). A thin bacterial biofilm was also observed on the root surfaces after 12 h, particularly in the root hair zone (Fig. 1b). Bacteria were also seen colonizing as a biofilm in the lateral root emergence zone, but in higher densities than was observed on root surfaces (Fig. 1c). No bacteria were detected on the tip root (data not shown). Despite the surface of the aerial parts being apparently free of bacteria, small aggregates were detected in the stomatal complexes by 24 h (Fig. 1d).

The presence of *Enterobacter* sp. UYSO10 colonizing the intercellular spaces was detected in the roots (Fig. 2a) and in the stem bases from 4 h after inoculation. In the lower stem, bacteria were present in the intercellular spaces of parenchyma cells (Fig. 2b–e). Occasionally, intercellular bacteria seem to be surrounded by a membrane-like structure (Fig. 2c), but this observation was not confirmed by TEM. Frequently, the presence of the bacteria was associated with an enlargement of the intercellular spaces (Fig. 2 b, c), which could be related to the production of cell wall-degrading enzymes. By 12 h, the bacteria were

Table 1 Effects of inoculation with putative bacterial endophytes on micropropagated sugarcane cv. LCP 85384

Treatment[a]	Stem diameter (mm)	Stem height (cm)	Dry weight (g plant^{-1})		N concentration (mg N g^{-1} dry weight)	N accumulation (mg plant^{-1})
			Roots	Shoot		
Negative control	5.09a[b]	8.14a	1.29a	0.72a	0.83a	0.55a
Acinetobacter sp. UYSO03	5.77ab	9.64abc	1.93bc	1.08abc	N.D.	N.D.
Enterobacter sp. UYSO10	6.40b	10.18bcd	1.6ab	1.46c	0.78a	1.16b
Pantoea sp. UYSO13	5.29ab	10.34bcd	1.82abc	0.97ab	N.D.	N.D.
Pseudomonas sp. UYSO14	5.36ab	9.04ab	1.75ab	0.93a	N.D.	N.D.
Rhanella sp. UYSO22	5.97ab	10.14bcd	1.71ab	1.09abc	N.D.	N.D.
Shinella sp. UYSO24	6.19ab	11.13d	2.34c	1.37bc	0.75a	1.06b
MIX	5.70ab	10.42bcd	1.85abc	1.11abc	N.D.	N.D.
G. diazotrophicus Pal5	5.97ab	10.73cd	1.75ab	1.10abc	N.D.	N.D.

MIX inoculum made with a mixture of all strains tested, *N.D.* not determined

[a] Negative controls uninoculated plants that were without N fertilization

[b] Means within two treatments that have the same letters are not significantly different by the Tukey 0.05 test

observed colonizing the lumen of the root metaxylem (Fig. 2f) and, by 24 h, the stem xylem vessels in high numbers (Fig. 2g, h). Bacteria were also observed colonizing the intercellular spaces of the leaves and the sub-stomatal cavities (Fig. 2i). In both roots and aerial parts, the DNA bacterial copy number was measured at 10^7 and 10^6 100 mg^{-1} of fresh tissue by 48 h, and this had increased, in the aerial part, to 10^7 100 mg^{-1} by 6 dpi (Fig. 3).

A plant reaction in the form of a gum was detected as a pink-violet-stained material at 6 h after inoculation in inoculated plants. This plant reaction was observed in both the roots and stems, in the xylem vessels, and the intercellular spaces. Interestingly, the plant reaction was observed regardless of the presence or absence of bacteria (Fig. 2b, j).

In a few cases, bacteria were observed in clumps accumulated on the surface of the wound caused by the separation of the plantlets at the start of the inoculation experiment. These bacteria were also detected entering the tissue through the intercellular spaces, and occasionally via apparently disrupted cells (Fig. 2j). It is important to stress, however, that intercellular bacteria could only be seen within cells adjacent to the broken tissues, suggesting that this strain (*Enterobacter* sp. UYSO10) enters as an endophyte into sugarcane plantlets via natural or injury-induced openings, as reported by James et al. (2001) for infection by *G. diazotrophicus*.

Table 2 Potential plant interaction traits of putative endophyte bacteria associated with Uruguayan sugarcane cultivars

Isolates	Interaction and infection traits						
	BIO	B.MS	CEL	HCE	PRO	PER	LAC
Acinetobacter sp. UYSO03	+	ND	+	−	−	−	−
Enterobacter sp. UYSO10	−	−	+	−	−	−	−
Pantoea sp. UYSO13	−	ND	+	−	+	−	+
Pseudomonas sp. UYSO14	+	+/−	−	−	+	−	−
Rhanella sp. UYSO22	−	ND	+	−	−	−	−
Shinella sp. UYSO24	−	+	+	−	−	−	+

BIO biofilm formation in TY medium, *B.MS* biofilm formation in MS + exudates medium ("MS + exudates" refers to MS medium that was exposed to micropropagated sugarcane root secretions for 72 h.), *CEL* endoglucanase activity, *HCE* hemicellulase activity, *PRO* protease activity, *PER* peroxidase activity, *LAC* laccase activity, *ND* not determined
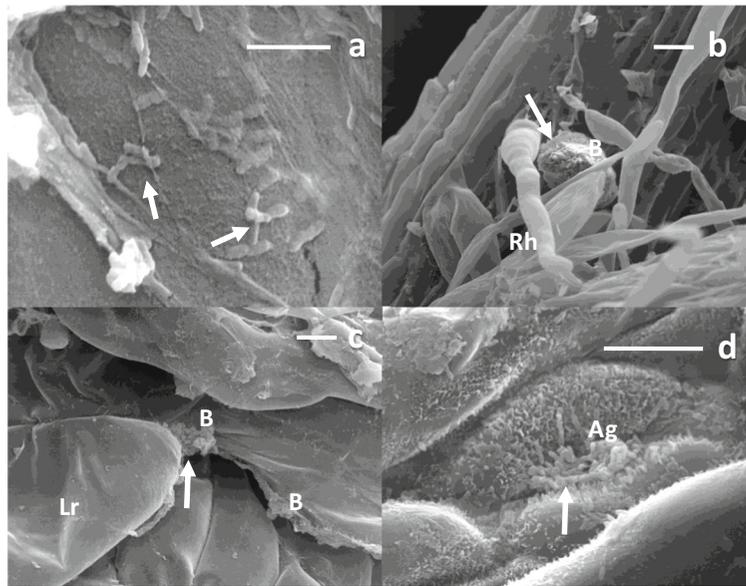
Fig. 1 Scanning electron micrograph (SEM) showing the surface colonization of micropropagated sugarcane by *Enterobacter* sp. UYSO10. **a** Single bacterial cells adhered in an apolar manner to the root and the base of the stem by 6 h after inoculation (*bar* = 5 μm). **b** Biofilm on the root hair zone by 24 h after inoculation (*bar* = 50 μm). **c** biofilm on the crack generated by the emergence of secondary roots by 24 h after inoculation (*bar* = 25 μm). **d** Bacterial aggregates in the stomata at 24 h after inoculation (*bar* = 10 μm). The bacteria are indicated with *white arrows*. *Ag* aggregate, *B* biofilm, *S* stem, *Rh* root hair, *Lr* lateral root

Colonization of sugarcane cv. LCP 85384 by *Shinella* sp. UYSO24

A profuse colonization of the root hair zone was observed by *Shinella* sp. UYSO24. The colonization was detected as aggregates as well as in biofilms by 12 h after inoculation, and these contained large numbers of bacteria, which were adhered to the root surface (Fig. 4a). In particular, bacteria were observed in association with meristematic tissues, such as the root cap and the points of lateral root emergence (Fig. 4b, c). On the other hand, the lower stem surface was colonized earlier (by 4 h after inoculation) by individual cells that were attached in a non-polar manner (data not shown, but similar to Fig. 1a), and by 12 h, bacteria were observed forming small biofilms on the lower stem surface (Fig. 4d).

The presence of *Shinella* sp. UYSO24 within the tissues was low and discrete, and it was rarely observed colonizing intercellular spaces (Fig. 5a–d). The colonization of the xylem vessels was first detected at 12 h after inoculation, mainly on the stem, and by single cells or small aggregates (Fig. 5e, f). The stomatal surface and sub-stomatal cavities were devoid of bacteria (data not shown). A plant reaction was detected within intercellular spaces in the root cortex and in some of the vascular tissue, but this was not necessarily associated with bacteria (data not shown). Although only a few bacteria were observed by microscopy, the DNA bacterial copy number was 10^7 and 10^6 per 100 mg of fresh tissue by 48 h after inoculation for the roots and aerial tissue, respectively (Fig. 3). By 6 dpi the root population had decreased, but the aerial tissue population was maintained. These results are in agreement with those obtained by bacterial plate counting (data not shown).

Control and reference treatments

No bacteria were detected by microscopy on the surface or within uninfected control plants (data not shown). In the case of the two reference treatments, the surface colonization was checked at 24 h after inoculation: with *G. diazotrophicus*, a known endophytic bacterium in sugarcane, roots, particularly the root tips, were colonized by single cells and by cell aggregates (Fig. 6a), whereas with *Pseudomonas* sp. UYSO14, which was used in this study as a reference strain because its inoculation did not promote sugarcane growth (Table 1), only individual bacteria were detected in the root hair zone and on the aerial tissue (Fig. 6b).
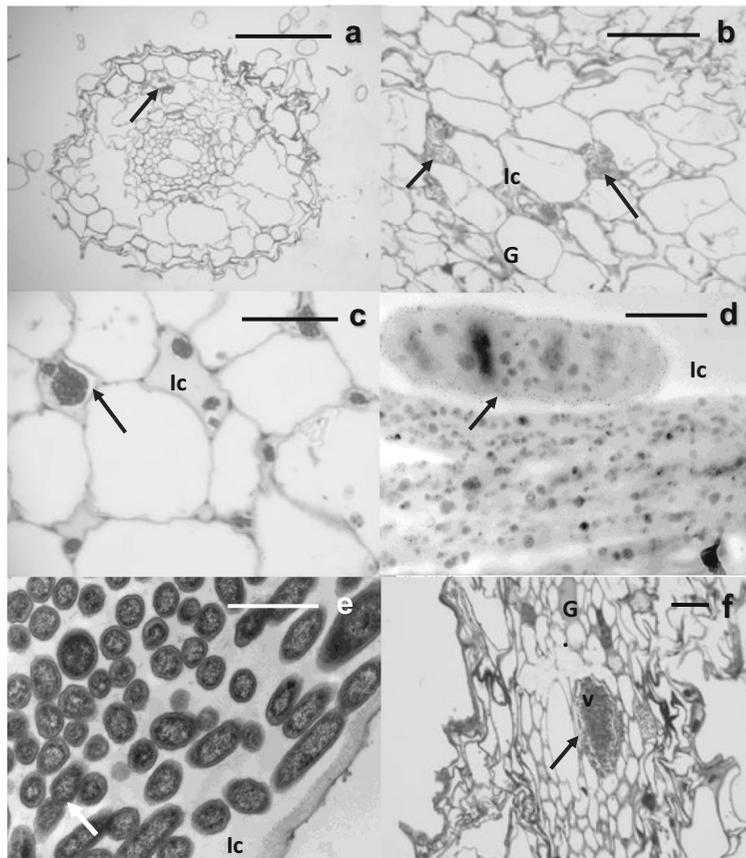
Fig. 2 Light microscopy (LM) and transmission electron microscopy (TEM) of transverse sections showing the inner colonization of micropropagated sugarcane tissues by *Enterobacter* sp. UYSO10. **a** LM of root intercellular space colonization at 6 h after inoculation (*bar* = 10 μm). **b**, **c** LM of stem intercellular space colonization by 12 h after inoculation (*bar* = 50 μm) (**b**) and (*bar* = 25 μm)(**c**). **d** TEM of stem intercellular space colonization at 48 h after inoculation with bacteria inmunogold labeled with an antibody against UYSO10 (*bar* = 200 nm). Note that the gold particles are attached to the perimeter of the bacterial cell. **e** TEM of stem intercellular space colonization at 48 h after inoculation (*bar* = 500 nm). **f** LM of root vascular tissue colonization at 12 h after inoculation (*bar* = 20 μm). **g** LM of stem vascular tissue colonization at 24 h after inoculation (*bar* = 20 μm). **h** Immunogold labeling (plus silver enhancement) with the antibody against UYSO10 (*bar* = 20 μm). **i** LM showing colonization of leaf intercellular spaces and a sub-stomatal cavity at 6 dpi (*bar* = 50 μm). **j** Bacterial colonization of wounded tissue caused by separation of micropropagated plantlets at 12 h after inoculation (*bar* = 20 μm). The bacteria are indicated with *arrows. G* gum, *Ic* intercellular space, *Sc* sub-stomatal cavity, *v* vascular tissue

No amplification of bacterial DNA was obtained by qPCR of uninoculated control plants, when specific primers for UYSO24 and UYSO24 were used.

Discussion

Enterobacter sp. UYSO10 and *Shinella* sp. UYSO24 promote the growth of micropropagated sugarcane

Many diazotrophic bacteria have been isolated from and/or detected in different sugarcane genotypes grown in several regions of the world (Cavalcante and Dobereiner 1998; Olivares et al. 1996; James and Olivares, 1998; Thaweenut et al. 2011; Burbano et al. 2011; Fischer et al. 2012; Taulé et al. 2012; Beneduzi et al. 2013). In addition, the PGP effects on sugarcane of associated or endophytic bacteria such as *G. diazotrophicus*, *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Azospirillum amazonense*, and *Burkholderia* spp., are well reported (James et al. 1994; Sevilla et al. 2001; Oliveira et al. 2002, 2006, 2009). The PGP effects observed depend on the biotic and abiotic conditions, as well as on the specificity and compatibility of the plant-bacterial
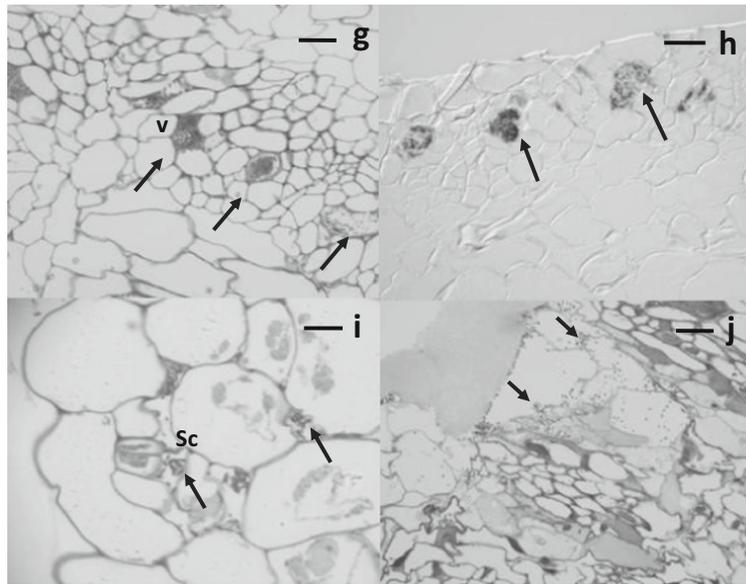
Fig. 2 continued.

genotypes (Reis Junior et al. 2000; Govindarajan et al. 2006; Carvalho et al. 2011).

For cv. LCP 85384, the best PGP strains for almost all of the parameters evaluated were *Shinella* sp. UYSO24 and *Enterobacter* sp. UYSO10. Considering

that both strains were reported as being both diazotrophic and auxin producers (Taulé et al. 2012), and that an increase in N accumulation was observed in the inoculated plants, we can reasonably speculate that part of the observed PGP effect could have come from
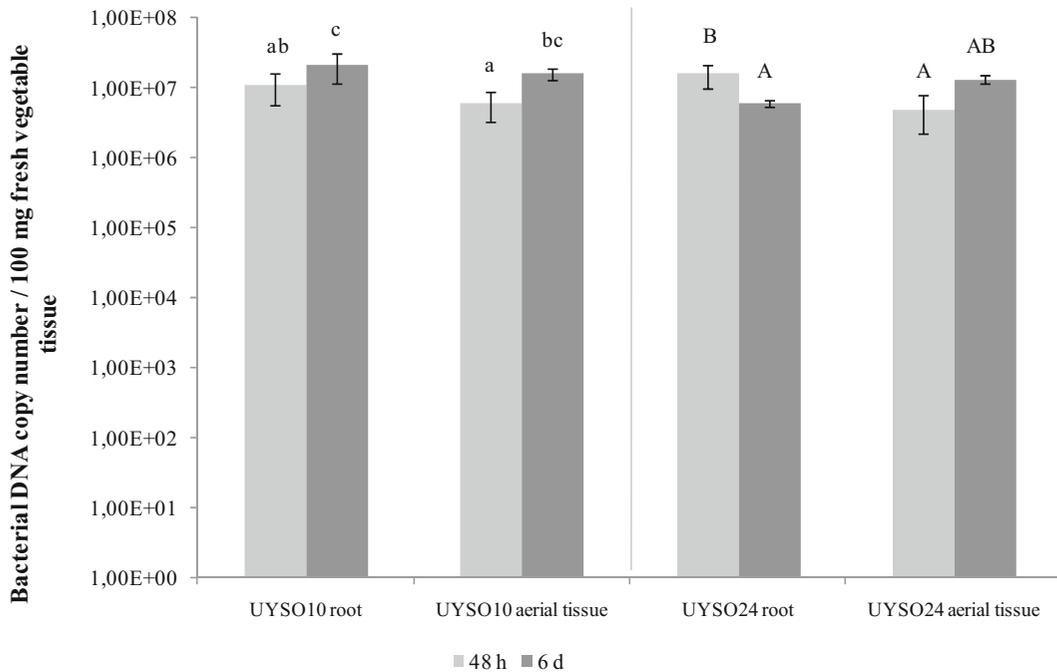
Fig. 3 Quantification by quantitative real time PCR (qPCR) of endophytic bacteria population colonizing micropropagated sugarcane cv. LCP 85384, at 48 h and 6 days after inoculation. Means within two treatments that have the same letters are not significantly different by the Tukey 0.05 test

Fig. 4 SEM showing the surface colonization of micropropagated sugarcane by *Shinella* sp. UYSO24 by 24 h after inoculation. **a** Biofilm layer on the root hair zone (*bar* = 50 μm), **b** biofilm formation on the emerging secondary root (*bar* = 50 μm), **c** on the root tip (*bar* = 50 μm), and **d** biofilm formation on the aerial tissue surface at 12 h after inoculation (*bar* = 10 μm). *White arrows* indicate bacteria. *B* biofilm, *Rh* root hair
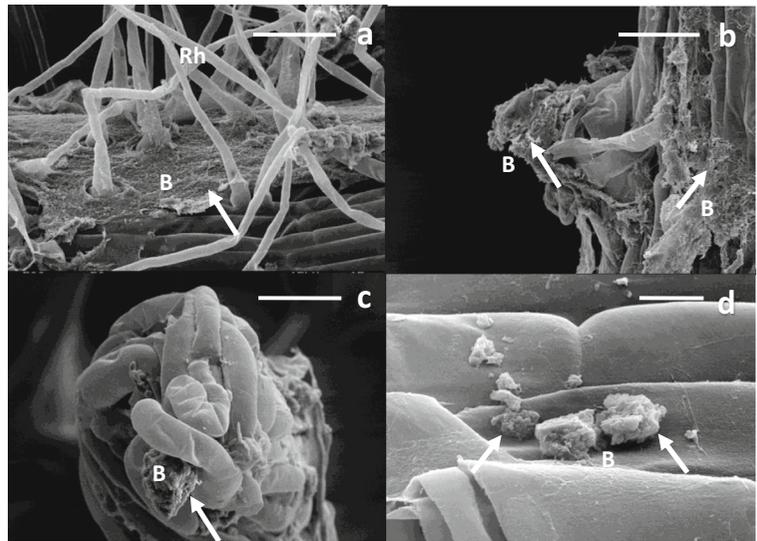
the BNF process, although further experiments are needed to confirm this. To our knowledge, this is the first report of a putatively endophytic PGP strain from sugarcane within the genus *Shinella*, but in contrast, several strains of *Enterobacter* have been reported as

PGPRs of rice (*Oryza sativa*), maize (*Zea mays*), and sugarcane (Taghavi et al. 2010; Keyeo et al. 2011; Naveed et al. 2014).

It should be noted that the reference strain *G. diazotrophicus* Pal5, which was used in the PGP

Fig. 5 LM and TEM of transverse sections showing the inner colonization of micropropagated sugarcane tissues by *Shinella* sp. UYSO24. **a** LM of root intercellular space colonization by 24 h pi (*bar* = 10 μm). **b**, **c** TEM of stem intercellular colonization by 12 h pi (*bar* = 1 μm) (**b**) and 48 h pi (*bar* = 2 μm). **d** TEM of a structure in an intercellular space that that is immunogold labeled with the antibody against *Shinella* sp. UYSO24 (*bar* = 500 nm). **e**, **f** LM of stem vascular tissue colonization by 48 h (*bar* = 25 μm) and 12 h (*bar* = 10 μm) (**f**). The bacteria are indicated with *black arrows*. *Ic* intercellular space, *v* vascular tissue
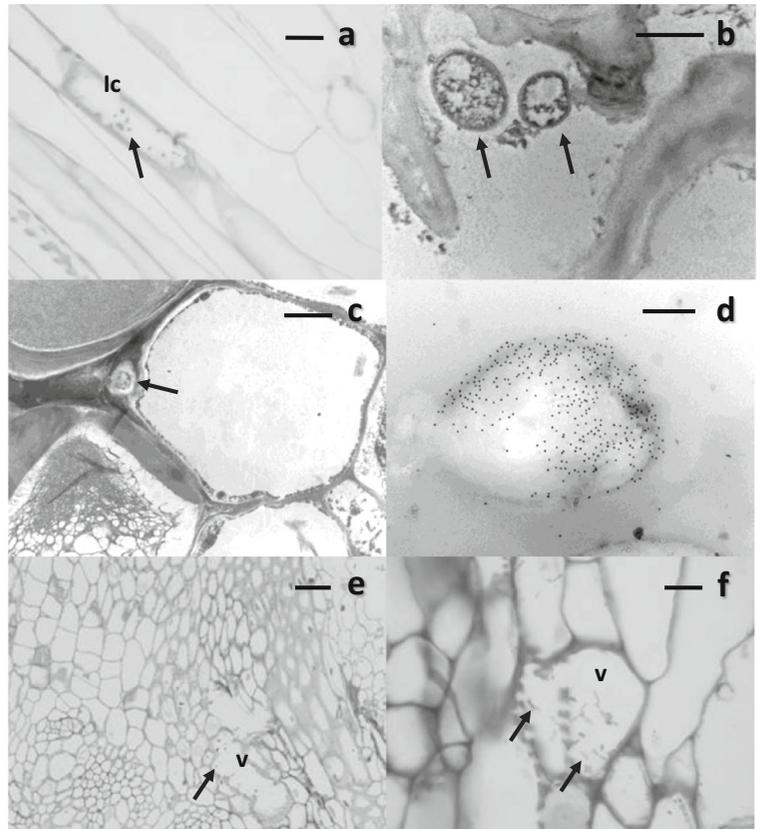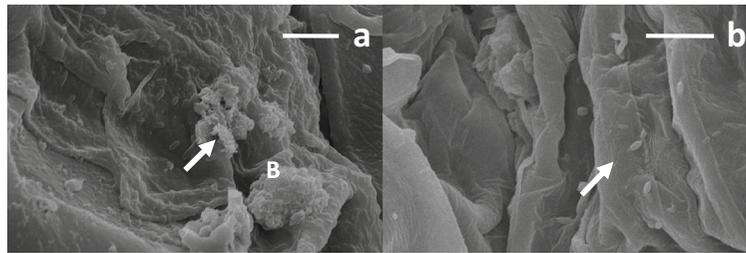
Fig. 6 SEM showing the surface colonization of micropropagated sugarcane by reference strains at 24 h pi. a *Gluconacetobacter diazotrophicus* Pal5 colonizing the root tip (*bar* = 5 μm). b

Pseudomonas sp. UYSO14 colonizing the aerial tissue as single cells (*bar* = 10 μm). The bacteria are indicated with *white arrows*, *B* biofilm

assay, did not perform as well as expected on cv. LCP 85384. It has been reported to be a sugarcane endophyte and the benefits from its inoculation onto sugarcane are well documented (James et al. 1994; Sevilla et al. 2001). Moreover, in a previous study, we were unable to isolate *G. diazotrophicus* despite using the specific methodology and media recommended for isolating it (Taulé et al. 2012). Taken together, the absence of *G. diazotrophicus* in cv. LCP 85384 was most likely due to the biotic and abiotic features of the crop location, to N fertilization effects, and because of this particular sugarcane genotype (Fierer and Jackson 2006).

With the aim of further understanding how these endophytic bacteria could interact with their host plant (sugarcane in this case), the strains used as inoculants were further characterized in vitro for their plant interaction traits. Hydrolytic enzymes play a key role in plant-pathogen and legume-rhizobium interactions, as well as in biocontrol (Rosenblueth and Martínez-Romero 2006; Robledo et al. 2008; Monteiro et al. 2008; Vacheron et al. 2013; Naveed et al. 2014). In the particular case of endophyte-plant interactions, the importance of an endoglucanase (EglA) in the ability of *Azoarcus* sp. BH72 to infect rice roots was demonstrated by Reinhold-Hurek et al. (2006), and the presence of endoglucanases has also been reported in other endophytic and/or plant-associated bacteria, including *Azospirillum* spp. and *Herbaspirillum* spp. (James et al. 2002; Lodewyckx et al. 2002; Monteiro et al. 2012). Accordingly, several hydrolytic enzyme activities, such as cellulase and protease were detected in vitro in the strains used in this study, as well as their ability to form biofilms. These traits could be involved in the plant-bacterial interaction, although further experiments are needed to demonstrate this. However, it is certainly significant that *Shinella* sp. UYSO24 formed biofilms in the presence of sugarcane exudates, this is an important

trait as successful and enduring endophytic interactions with a plant are most likely mediated via signals from the host

Enterobacter sp. UYSO10 and *Shinella* sp. UYSO24 are endophytes of sugarcane

The plant surface colonization behavior of both strains was similar to other non-pathogenic endophytic bacteria (James et al. 1994, 2001; Hallmann et al. 1997; Reinhold-Hurek and Hurek 1998; James et al. 2002). For both strains studied, the main colonization sites observed were the cracks formed by the emergence of the lateral roots, on the root hair zone, as well as on the root tips. Interestingly, large differences in surface colonization were observed between both strains in the type of biofilm produced. *Enterobacter* sp. UYSO10 formed small spherical biofilms, principally located in the root hair zone, while *Shinella* sp. UYSO24 form extended laminar biofilms that were localized in association with meristematic tissues as well as in the root hair zone.

The colonization of sugarcane cv. LCP 85384 by the strains employed as references in the plant-bacterial interaction experiments showed that the surface and endophytic colonization by *G. diazotrophicus* was similar to that reported in other sugarcane cultivars (James et al. 1994, 2001; Reis et al. 1999; Sevilla et al. 2001), albeit somewhat reduced, which is most likely due to cv. LCP 85384 not being well matched with this particular endophytic diazotroph. In contrast to *G. diazotrophicus*, and to both of the test strains, *Pseudomonas* sp. UYSO14 only colonized the root surface as single cells, and neither aggregates nor biofilms could be detected; the low colonization might be related to the absence of an observed PGP effect by this strain.

As both strains were able to colonize the inner tissues of sugarcane, they can be defined as endophytes. For

example, *Enterobacter* sp. UYSO10 extensively colonized the inner tissues of sugarcane, entering the roots, stems and leaves, including the vascular tissue. Although no direct evidence that the bacteria could penetrate the endodermis was obtained, they were detected colonizing the vascular system in high numbers, which has been reported for other endophytes in sugarcane (James et al. 1994, 2001; Olivares et al. 1997). This is also the case with *H. seropedicae*, which colonize the xylem of rice in high numbers, although by 14 dpi the presence of bacteria in the vascular tissues decreases (James et al. 2002). *Enterobacter* sp. UYSO10 was also detected colonizing the sub-stomatal cavities and the exterior of the stomata, suggesting them as an entrance or exit point for the bacteria. Indeed, the leaves might be colonized by this strain via vascular transport, suggesting a systemic distribution. Similar observations were made for *H. seropedicae* and *H. rubrisubalbicans* which colonizes the leaves of sugarcane and sorghum in the early stages of infection, and which was suggested to be related to the high numbers of bacteria present in the vascular tissue, which would then allow for their translocation to other organs (James et al. 1997; Olivares et al. 1997). Moreover, when *G. diazotrophicus* and *H. seropedicae* were inoculated onto rice or sugarcane the presence of the bacteria in the sub-stomatal cavity was also reported (James et al. 2001, 2002), and was thus suggested that the plants regulated the number of bacteria by ejecting some of them via the stomatal complex.

qPCR data showed that the numbers of cells of both the inoculated strains were of the same order as those of other bacterial endophytes reported to be colonizing plants in vitro (Ruppel et al. 2006; Couillerot et al. 2010; Pellizzaro Pereira et al. 2014). In the case of *Enterobacter* sp. UYSO10, the number of bacteria increase after 6 dpi, which correlated with the observations made in the microscopy experiments.

Several strains in the genus *Enterobacter* have been described as endophytes of a variety of plants. These possess several in vitro PGP and plant colonization traits, and some of them have also been described as PGPRs (Morales-Valenzuela et al. 2007; Taghavi et al. 2009; Madhaiyan et al. 2013; Naveed et al. 2014). Studies conducted to define them as endophytes describe similar colonization patterns to that reported here for strain UYSO10 (Quadt-Hallmann and Kloepper 1996; Quadt-Hallmann et al. 1997; Taghavi et al. 2009). Additionally, in vitro studies of *Enterobacter* sp. UYSO10 displayed endoglucanase activity, which

may be involved in entering the plant interior and in their subsequent dispersion *in planta*.

When *Shinella* sp. UYSO24 was inoculated onto micropropagated sugarcane plants, inner tissue colonization was low and discrete. The infection route mainly involved the colonization of the vascular tissue by single cells or by small aggregates. Additionally, a low presence of bacteria was observed in the intercellular spaces which may be due to a preference for other niches *in planta*. Therefore, considering the low bacterial numbers of bacteria observed within the inner tissues, it is most likely that the PGP effects observed for this strain came from the high number of bacteria colonizing the rhizoplane. However, against this, and in contrast to the observations made using microscopy of a low bacterial colonization of the inner tissues, bacterial quantification by qPCR suggested greater colonization by this strain. Further work is needed to help explain this apparent discrepancy between the two techniques. The case of *Shinella* sp. UYSO24 as a PGPR and, in particular, as an endophyte is very interesting as it is the first report of a strain belonging to this genus that has a positive effect on plant growth. The alphaproteobacterial genus *Shinella* was defined recently (An et al. 2006) and comprises six defined species, including *S. kummerowiae*, a non-nodulating endophyte of legume nodules (Lin et al. 2008), but there are several 16S rDNA genes sequences from this genus available in public databases that so far lack species characterization, which indicates that more work is needed to fully understand the ecological implications of this genus.

Concluding remarks

In this study, the interaction between the strains *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 with the commercial sugarcane cv. LCP 85384 are described. Both diazotrophic strains were isolated from surface-sterilized upper stems of healthy commercially grown sugarcane plants in Uruguay, and both were shown to be capable of promoting the growth of micropropagated plants of the same variety (LCP 85384) under greenhouse conditions. Taken together, these data emphasize their suitability for selection as potential candidates for any future inoculant formulations. Given that one of the most crucial steps for the biotechnological application of such inoculants is their root colonization (Pliego et al. 2011), it is of utmost

importance to understand how they interact with their host plants. Accordingly, the surface colonization pattern of both strains was described, and interestingly, was shown to differ between both endophytes, thus suggesting that there is no common strategy utilized by endophytic bacteria for colonizing sugarcane.

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