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Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization

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Abstract

Acetobacter diazotrophicus is a nitrogen-fixing endophytic bacterium, originally isolated from sugarcane. Its colonizing ability was evaluated in high and low N-fertilized sugarcane plants by inoculating stem-cuts with a β-glucuronidase marked *A. diazotrophicus* strain. Bacterial quantification by the most probable number technique showed a severe decrease of *A. diazotrophicus* cells in plants fertilized with high levels of nitrogen. The inoculated strain was detected inside low N-fertilized sugarcane plants by histochemical staining of β-glucuronidase and scanning electron microscopy. *A. diazotrophicus* was found mainly inside cortical cells of stems and inside xylem vessels. No β-glucuronidase activity was observed in non-inoculated plants. High nitrogen fertilization of fields might be a threat to maintaining naturally occurring endophytic associations.

Keywords: *Acetobacter*; Auxin; Endophyte; Nitrogen-fixing bacteria; Sugarcane; Xylem

1. Introduction

The sugarcane crop is vegetatively propagated by use of stems and this plant produces large amounts of biomass which demand a massive input of nutrients, especially N and K [1]. In almost all countries where this crop is cultivated, a common agricultural practice is to apply 250 kg N or more per Ha. Nevertheless, Brazilian farmers have used amounts of fertilizer that do not adequately cover the theoretical loss of nitrogen occurring when the plants are harvested. Surprisingly, these crops do not show nitrogen deficiencies, and their response to the addition of nitrogen fertilizer is usually negligible [2]. Consequently, biological nitrogen fixation (BNF) has been suggested to contribute to the nutrition of sugarcane plants [3]. In fact, experiments using ^{15}N isotope dilution or N balance methods gave evidence that BNF provided an important proportion of the nitrogen requirements of different sugarcane varieties [4,5].

Different N_2-fixing bacteria, such as *Enterobacter cloacae*, *Erwinia herbicola*, *Klebsiella pneumoniae*,

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Azotobacter vinelandii, *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*), *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* and *Acetobacter diazotrophicus* colonize the sugarcane rhizosphere and inner tissues [3,6,7]. These bacteria, and possibly other diazotrophs not yet isolated could contribute to BNF in this plant. It appears that when the stalks are sown, they carry endophytic bacteria that may spread inside the plant after budding. *A. diazotrophicus* has been suggested to be an endophytic contributor of nitrogen to this crop, as it fixes nitrogen in culture medium under acidity levels and sugar concentrations that resemble those inside the plant [6,8,9]. It has been reported that the frequency of isolation of *A. diazotrophicus* from sugarcane plants diminishes in relation to the amounts of N-fertilization used in the fields [10,11]. Caballero-Mellado et al. [12] found that Brazilian isolates were genetically more diverse than Mexican ones and suggested that this could be related to the difference in nitrogen fertilization levels between the two countries, in such a manner that the application of more fertilizer caused a diminished diversity. In the nitrogen-fixing symbiosis of *Rhizobium* and legumes, high nitrogen fertilization abolishes nodulation or, when applied to existing nodules, nitrogen fixation. It was therefore of interest to evaluate if the supposedly nitrogen-fixing *A. diazotrophicus*-sugarcane association was similarly affected by nitrogen.

By using microscopic techniques in root-inoculated sterile plantlets, James et al. [13] detected *A. diazotrophicus* colonizing the root intercellular spaces, and the interior of root epidermal cells. They proposed that *A. diazotrophicus* could be distributed from the base of the stem to other organs via the stem xylem vessels, since they also detected xylem colonization in the basal region of the stalk. In non-inoculated sugarcane plants, Dong et al. [14] isolated *A. diazotrophicus* from apoplastic fluid, that includes fluid from various locations, such as cell walls, intercellular spaces, and xylem sap [15].

The aim of this work was to examine the effects of nitrogen fertilization on the endophytic colonization of the sugarcane by inoculating an *A. diazotrophicus gusA* marked strain. In addition, we attempted to clarify the location of *A. diazotrophicus* inside the plant.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strains were cultured in Luria–Bertani medium at 37°C. When necessary kanamycin (Km), streptomycin (Sm), spectinomycin (Sp), and nalidixic acid

Table 1
Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli		
CMK	Nal^s, Sm^s	[41]
A. diazotrophicus		
UAP 5541	Wild-type, without plasmids, able to fix N_2 in vitro, common clone by multilocus assay, Nal^r	[10,18]
Plasmids		
pRK2013	Km^r, Tra^+, ColE1 replicon, helper plasmid	[42]
pRG960SD	Sm^r, Sp^r cosmid, IncP, Mob^+, promoter-less *gusA* with a Shine and Dalgarno sequence	[17]
pBI426	Ap^r, Km^r vector with *gusA*-NPTII expressed from a double 35S CaMV virus promoter plus a leader sequence from alfalfa mosaic virus, also *gusA*-NPTII expressed from unidentified region in different Gram-negative bacteria	[16]
pRGS561	Sm^r,Sp^r, pRG960SD derivative with *gusA*-NPTII constitutive expression in *A. diazotrophicus*	This work

[a]Nal, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; Km, kanamycin; Ap, ampicillin; s, sensitive; r, resistant.

(Nal) were added at final concentrations of 25, 60, 60, and 15 μg ml^{-1}, respectively. LGI medium [6] was used for growing *A. diazotrophicus* strains, and N-free semi-solid LGI [6] for the isolation of the inoculated strains. For triparental conjugations, MESMA medium with the following composition was used (g l^{-1}): yeast extract, 2.7; glucose, 2.7; mannitol, 1.8; MES (Sigma, St. Louis, MO), 4.4; K_2HPO_4, 4.81; KH_2PO_4, 0.65; Bromothymol blue, 0.025; and agar, 12; pH 6.7. For measuring the GUS activity of *A. diazotrophicus*, cells were grown in LGI broth, containing (g l^{-1}): K_2HPO_4, 0.2; KH_2PO_4, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeCl_3 \cdot 6H_2O$, 0.01; $Na_2Mo_4 \cdot 2H_2O$, 0.002; and $(NH_4)_2SO_4$, 0.617; plus the carbon source previously filter-sterilized (sucrose, fructose, glucose or gluconate, 1.5%), pH 5.5. GUS activity was also determined in cells growing with sucrose (10%) added to semi-solid SUCMES, containing (g l^{-1}): MES 4.4, K_2HPO_4 4.81, KH_2PO_4 0.65, $(NH_4)_2SO_4$ 1.48, and agar 1.5, pH 6.7.

2.2. Plasmids and strain construction

To construct a *gusA* marked strain, a DNA fragment from pBI426 [16] carrying an alfalfa mosaic virus leader sequence and a double 35S CaMV promoter fused to *gusA*-NPTII, was inserted into the broad-host range plasmid pRG960SD digested with *Eco*RI–*Hin*dIII [17]. The resulting construct (pRGS561) was conjugatively mobilized from *E. coli* to *A. diazotrophicus* strain UAP 5541 [10,18] by triparental mating using *E. coli* HB 101/pRK2013 as a helper. *A. diazotrophicus* transconjugants were selected on MESMA plates containing Nal (15 μg ml^{-1}) and Sm (45 μg ml^{-1}).

2.3. Fluorogenic β-glucuronidase assays

The β-glucuronidase (GUS) activity of *A. diazotrophicus* UAP 5541 carrying pRGS561 was tested in vitro with a fluorogenic assay as described by Jefferson [19]. The wild-type strain *A. diazotrophicus* UAP 5541 and its derivative were grown with different carbon sources at 1.5% concentration (sucrose at 1.5 and 10%). Bacterial cells were resuspended in extraction buffer containing 50 mM sodium phosphate, pH 7.0; 10 mM β-mercaptoethanol; 10 Mm Na_2EDTA; 0.1% *N*-lauroylsarcosine; and 0.1% Triton X-100. Bacterial extracts were incubated at 37°C in MUG buffer, consisting of 1 mM 4-methylumbelliferyl-D-glucuronide (Sigma, St. Louis, MO) in extraction buffer. Aliquots were removed every 5 min for 30 min. The reaction was stopped by mixing aliquots with 0.2 M Na_2CO_3. For each assay, a calibration curve was preformed with 100 nM 4-methylumbelliferone (MU) in extraction buffer. Fluorescence determinations were performed with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA), at wavelengths of 365 nm (excitation) and 460 nm (emission).

2.4. Inoculation and growth of plants

Adult stalks of the sugarcane varieties Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418, regenerated from tissue cultures and subsequently grown in experimental fields, were kindly supplied by R. Méndez-Salas (Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Zacatepec, Mexico). This plant material was selected for the colonization experiments as it was shown to lack endogenous β-glucuronidase activity.

A. diazotrophicus strains were introduced inside sugarcane stems (setts) prior to their budding so as to resemble the *A. diazotrophicus*–sugarcane relationship under natural conditions. Setts having one node were inoculated with bacteria suspended in water. The setts were previously dehydrated at 45°C for 8–10 h. Approximately 10^7 bacterial cells were inoculated per plant. The setts were planted in sterile humid vermiculite/perlite mixture (1:1), incubated in a greenhouse at 28°C, and watered with sterile water until they began to bud. After budding, 50 ml of MS modified mineral solution [20] was added weekly per plant, for ten times maximum. The mineral solution contained the following: 1.5 mM $MgSO_4 \cdot 7H_2O$, 100 μM H_3BO_3, 30 μM $ZnSO_4 \cdot 7H_2O$, 100 pM $CuSO_4 \cdot 5H_2O$, 5.3 μM KI, 105 pM $CoCl_2 \cdot 6H_2O$, 1 μM $Na_2MoO_4 \cdot 2H_2O$, 100 mM $MnSO_4 \cdot H_2O$, 3 mM $CaCl_2 \cdot 2H_2O$, 4.1 mM Na_2EDTA, 6.7 mM $FeSO_4 \cdot 7H_2O$, and 1.3 mM potassium phosphate, pH 6.0. Nitrogen fertilizer was supplied every 2 weeks, with the high and low treatments consisting of 11 and 0.56 mmol of NH_4NO_3 per plant, respectively. The plants used for histo-

chemical GUS assays, inoculated strain isolation and scanning electron microscopy were collected 1, 2, 3, 5 or 7 months after sprouting.

2.5. Histochemical β-glucuronidase analysis

The histochemical assay was done as recommended by Jefferson and Wilson [21]. Each plant sample from all the varieties tested was aseptically separated into two subsamples. Sections of stem and roots from one subsample were incubated in the following buffer: 2 mM X-Gluc (Biosynth, Staad, Switzerland), previously dissolved in DMSO, 100 mM sodium phosphate pH 7.0, 0.5 mM Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 10 mM Na_2EDTA, and 2 nM $Na_2S_2O_5$. Sections from the other subsample were incubated in the same buffer supplemented with 0.02% NaN_3. The sections were incubated for 24 h at 30°C and used for microscopical analysis.

2.6. Scanning electron microscopy

Plant samples were fixed in 3% glutaraldehyde in 100 mM sodium phosphate pH 7.0, washed with 100 mM sodium phosphate pH 7.0, fixed in 1% osmium tetroxide, washed again with phosphate buffer and predehydrated with increasingly concentrated ethanol (from 30 to 99%). The specimens were dehydrated to critical point and gold coated. Observations were carried out in a JSM-5410LV (Jeol, Tokyo, Japan) scanning electron microscope.

2.7. Optical microscopy

At each sampling time (10 days, 1, 2, 3, 5 and 7 months after sprouting) stems and roots of two plants assayed for GUS activity were observed under low magnification. Two sections from each stem of plants without GUS activity and two stem sections with GUS activity were selected for examination at higher magnifications. Samples were fixed in glutaraldehyde, washed in 100 mM sodium phosphate pH 7.0 and predehydrated with ethanol as described above. Samples were immersed in propylene oxide, and in 1:1 propylene oxide-Eponate 12 resin (Pelco, Redding, CA) mixture, and embedded in Eponate 12. Polymerization was carried out overnight at 60°C and 1.5 μm sections were used for observation. From the fixed samples three subsections were taken from two different non-inoculated and two inoculated plants which had been grown under two nitrogen fertilizer doses.

2.8. A. diazotrophicus re-isolation

A. diazotrophicus was isolated from inoculated sugarcane plants as described previously [10]. Small pieces from the plant samples to be assayed histochemically for GUS activity were aseptically separated and crushed for inoculation in LGI semi-solid media. After 6 days at 30°C the bacterial growth was streaked on LGI plates and incubated at the same temperature for 5 days.

2.9. Quantification of A. diazotrophicus cells

From sugarcane plantlets cv. Z MEX 5532 (1 and 2 months after spouting), *A. diazotrophicus* cells were quantified by the most probable number method (MPN). Root and stem samples obtained from surface sterilized sugarcane plants were finely macerated and resuspended in a chilled sucrose solution (1%). Serial dilutions were inoculated by triplicate in LGI semi-solid media containing cycloheximide (150 μg ml^{-1}) and incubated at 30°C for 5–6 days. Diazotrophs were enriched by incubating under similar conditions in the same media. Positive growth of *A. diazotrophicus* was determined by acidification and formation of the typical pellicle [6]. Numbers of bacteria were normalized to fresh weight of tissue. The presence of *A. diazotrophicus* was verified by morphology in LGI plates. In addition, Sm resistance in MESMA plates and GUS activity were confirmed in isolates from plants inoculated with UAP 5541 carrying pRGS561. The plasmid was purified from 15 colonies and observed by ethidium bromide staining.

2.10. Experimental design

Two different experiments were performed to evaluate the effect of the nitrogen on *A. diazotrophicus* colonization. For the first experiment, plants (cv. Z MEX 5532) were used 30 days after sprouting. The plants for the first experiment consisted of 20 canes

grown with the low nitrogen dose and 24 canes with the high nitrogen dose. The difference between the number of *A. diazotrophicus* in canes grown under low and high nitrogen fertilization 30 days after sprouting were tested with Student's *t*-test. For the second experiment, plants 60 days after sprouting (cv. Z MEX 5532) were used. For this determination, four inoculated plants grown with the low nitrogen fertilization and four grown with the high nitrogen fertilization were processed, as well as eight non-inoculated controls, four of which were grown under the high and four under the low fertilization conditions.

Additionally, colonization was detected by re-isolating *A. diazotrophicus* from inoculated plants 1, 2, 3, 5 and 7 months after sprouting. The varieties used in this experiment were Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418.

3. Results

3.1. Colonization of sugarcane by A. diazotrophicus

The inoculated strains could be recovered and identified from low N-fertilized sugarcane varieties Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418 at 1, 2, 3, 5 and 7 months after sprouting. Isolated bacteria produced the typical *A. diazotrophicus* growth in semi-solid LGI and showed the expected colony morphology in LGI plates. The numbers of *A. diazotrophicus* that endophytically colonized sugarcane stems or roots (cv. Z MEX 5532) fertilized with different nitrogen quantities showed significant differences (Table 2). Differences in *A. diazotrophicus* colonization was also seen in 60-day plants. The numbers in low N-fertilized plants were 2.9×10^3 and 1.2×10^3 colony forming units (CFU) per g of fresh weight in the stem and in the roots, respectively, while in the high N-fertilized plants, *A. diazotrophicus* was not detected by the most probable number method. Even in older plants (up to 7 months) of five different cultivars (Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418), *A. diazotrophicus* could not be isolated from inoculated plants maintained under high nitrogen fertilization, while isolation was always successful from plants maintained under low nitrogen fertilization (results not shown).

No *A. diazotrophicus* isolates were obtained from non-inoculated plants. An estimate of the total number of bacteria endophytically colonizing the roots and stems of sugarcane was in a range from 4×10^6 to 40×10^6 CFU per g of plant fresh tissue. These values were obtained from the bacterial growth in the semi-solid selective medium LGI. Possibly a great proportion of these bacteria were nitrogen-fixers, since the nitrogen in semi-solid LGI medium comes only from impurities in the components. Different types of bacteria were isolated in LGI media from non-inoculated plants. None of them corresponded morphologically to *A. diazotrophicus* in semi-solid LGI or LGI plates and we did not try to define their taxonomic status.

3.2. Localization of A. diazotrophicus in planta

To follow plant colonization by *A. diazotrophicus*, a $GusA^+$ strain was obtained by using a DNA fragment with the alfalfa mosaic virus leader and the double CaMV promoter fused to *gusA* [16]. The strain showed GUS activity in vitro when grown with carbon sources, such as gluconic acid, fructose, glucose and sucrose (results not shown).

GUS activity was detected in stems, but only in plants with low nitrogen fertilization (varieties: Z

Table 2
A. diazotrophicus cell numbers colonizing inoculated sugarcane plants at 30 days after sprouting[a]

	Stems	Roots
Low N[b]	$5.7\times10^{2\ A}$ (1.75×10^2)	$2.7\times10^{2\ A}$ (1.28×10^2)
High N[c]	$0.5\times10^{2\ B}$ (0.4×10^2)	$<0.3\times10^{2\ B}$

Superscript A and B mean that difference in bacterial numbers is significant ($P>0.05$).
[a]CFU per g of fresh weight determined by most probable number counting technique.
[b]Mean of 20 plants (S.E.M. in parentheses).
[c]Mean of 24 plants (S.E.M. in parentheses).

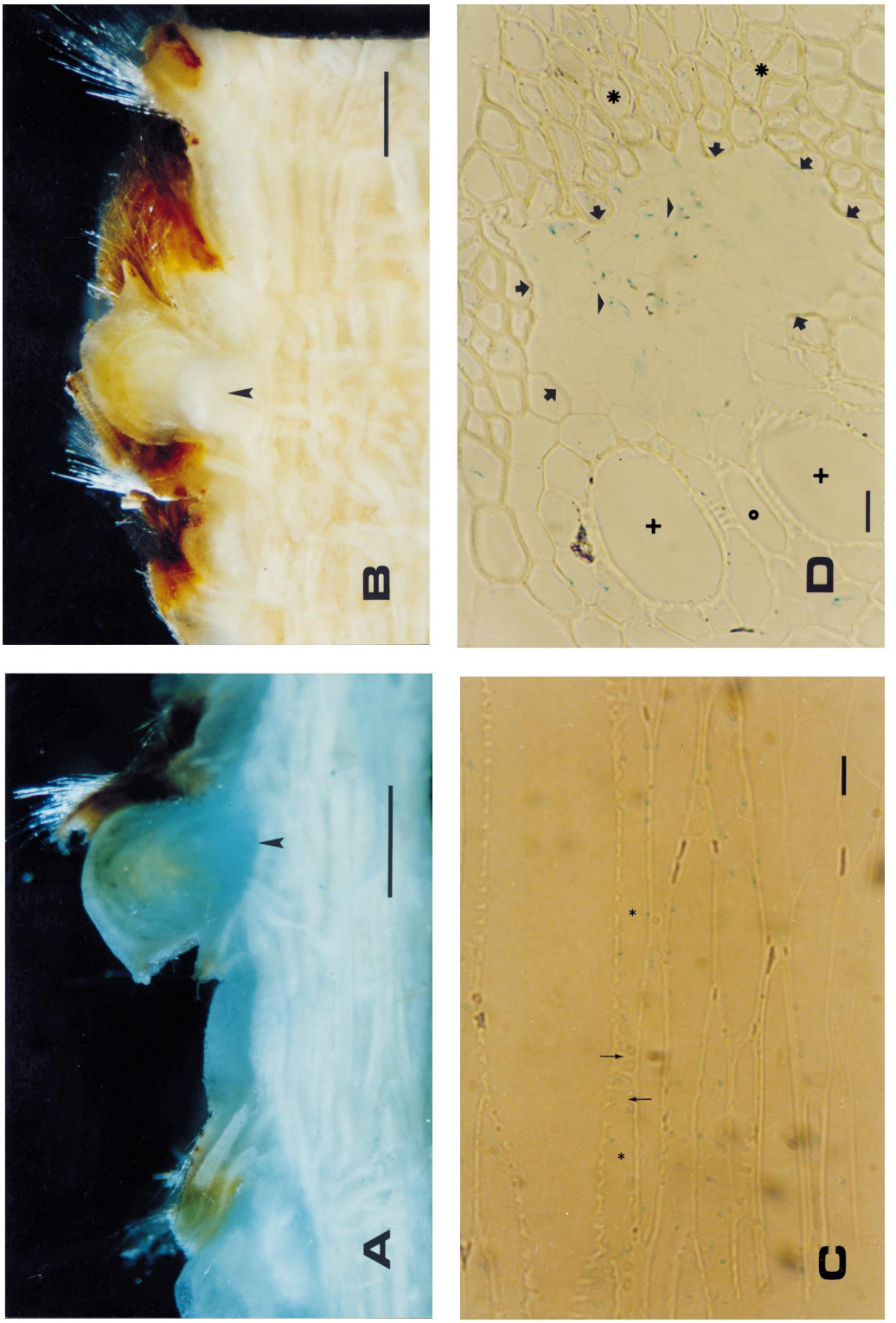

Fig. 1. GUS expression in sugarcane plants inoculated with a β-glucuronidase marked *A. diazotrophicus* strain, the GUS activity was detected with X-Gluc. Sections of stems of 2-month-old plants inoculated with the strain UAP 5541, carrying the plasmid pRGS561. (A,C,D) sections of stems of plants grown with low N-fertilization, and (B) section of a stem of a plant grown with high N-fertilization. (A,B) Low magnifications of longitudinal sections of inoculated sugarcane stems, developing buds are shown with arrows; (A) section of a stem showing GUS activity; (B) section of a stem with no GUS activity. (C) Longitudinal section of a vascular bundle showing a xylem vessel (*), the secondary wall growth of the vessel is shown with arrows. (D) Transversal section of a vascular bundle, two metaxylem vessels (+) a protoxylem (○) the sclerenchymatous sheath (*) and the phloem, delimited with arrows, are shown; the principal GUS activity is observed in phloem sieve tubes (▼). Scale bars: A and B, 0.5 mm; C and D, 20 μm.

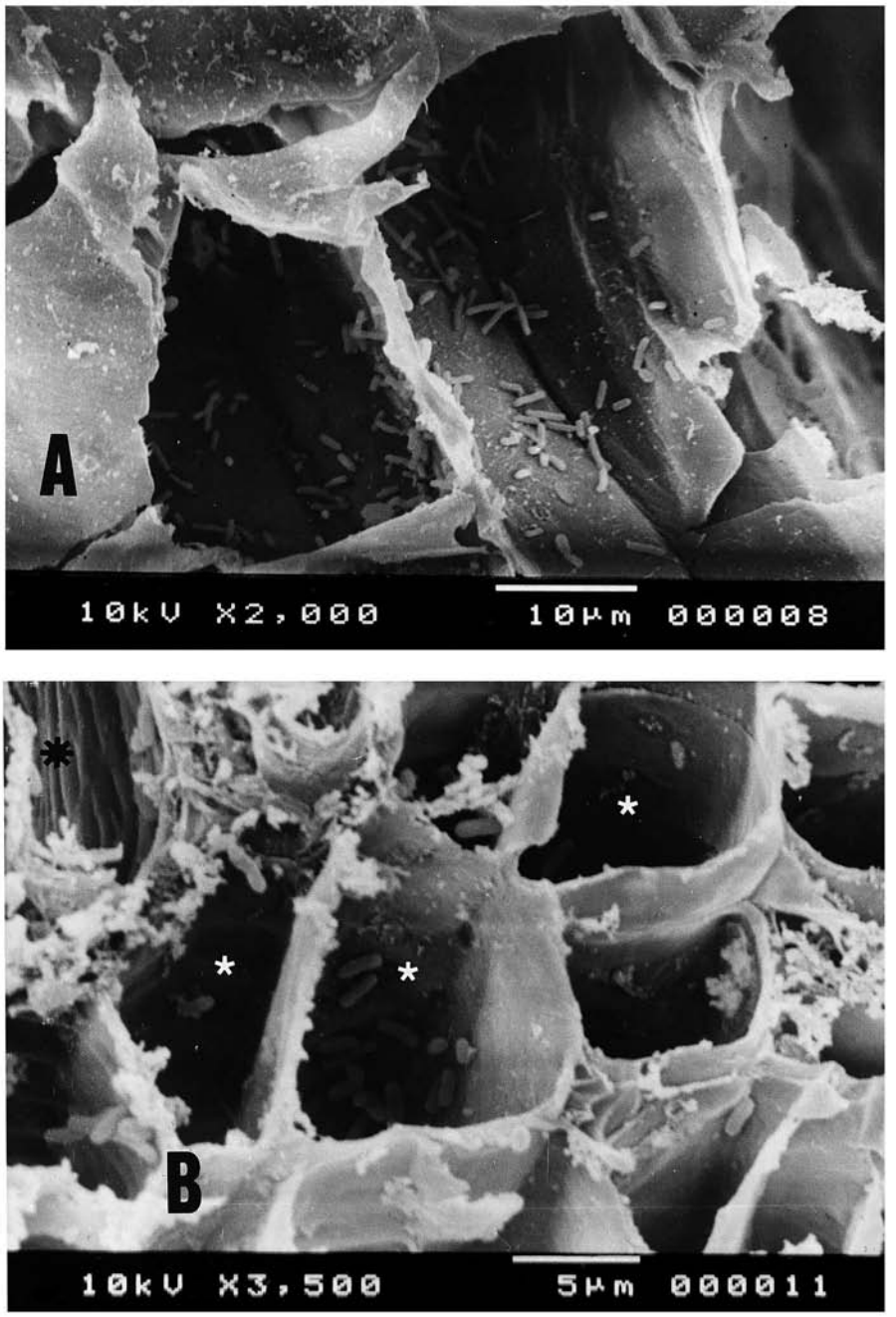

Fig. 2. *A. diazotrophicus*-like cells inhabiting cells of sugarcane stems. Sections of 5-month-old plants grown with low N-fertilization. (A) Section of sucrose storage parenchymatous cells located near to the stem cortex. (B) Section of a vascular bundle, a tracheary element (black asterisk) is surrounded by parenchyma cells (white asterisks).

MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418). Only in assays without NaN_3 was GUS activity detected. In stem tissues, the highest GUS activity was present in the cortex and the vascular bundle (xylem vessels and apparently also phloem sieve tubes), (Fig. 1A,C,D). GUS activity was never observed in non-inoculated plants (not shown). Scanning microscopy of stem samples from plants inoculated with the strain UAP 5541 carrying pRGS561 showed bacterial cells adhering to the walls of plant cells (Fig. 2). These bacteria were morphologically indistinguishable from typical *A. diazotrophicus* rods (0.6×2 μm). The vascular bundle and its surrounding cells were most abundantly colonized by these cells. In the non-inoculated controls, it was also possible to observe bacterial cells (not shown), but they were clearly different in size and shape from the *A. diazotrophicus* cells.

4. Discussion

The internal colonization of sugarcane by *A. diazotrophicus* in plants maintained with low and high nitrogen fertilizer doses was evaluated with a *gusA* Sm^r strain by re-isolation of the strain, confirmation of its identity by histochemical staining for β-glucuronidase and antibiotic resistance, and by scanning electron microscopy.

We used plants originally regenerated from tissue culture because they lacked endogenous GUS activity. GUS activity was obtained from stems of different sugarcane varieties coming from agricultural fields, and it is most probably of bacterial origin, as described in *Dioscorea* [22]. Successful colonization was observed in all the sugarcane varieties used, but the inoculated strain and the GUS activity were only detected in plants grown under low levels of nitrogen fertilization. The lack of *A. diazotrophicus* colonization in the presence of high supplied nitrogen explains the low presence and low frequency of isolation of *A. diazotrophicus* from sugarcane plants grown in fields with high nitrogen fertilization levels, reported previously by Fuentes Ramírez et al. [10] and Muthukumarasamy [11]. In experiments with the grasses *Miscanthus sinensis*, *M. sacchariflorus* and *Spartina pectinata*, Kirchhof et al. [23] found similar results by quantifying the cell numbers of the diazotrophic endophytic community inhabiting plants fertilized and unfertilized with nitrogen. This effect of nitrogen on colonization might not be universal since, for instance, *Herbaspirillum rubrisubalbicans* behaves as a pathogen in susceptible sugarcane cultivars grown in countries where high levels of nitrogen fertilization are used [7]. We detected 10^2–10^3 CFU per g of fresh tissue of *A. diazotrophicus* endophytically colonizing low N-fertilized 30-day-old sugarcane plants. In roots of non-inoculated sugarcane plants, Reis et al. [24] found 10^4–10^6 CFU of *A. diazotrophicus* per g of fresh tissue. The quantitative difference found between that report and ours could be related to the inoculation process that we used. In addition, the estimate of Reis et al. [24] could also include superficially adhering cells in addition to endophytic ones, and the plant cultivar might also have an influence [15]. In a study of the association of *A. diazotrophicus* with different cultivars of sugarcane, da Silva et al. [25] suggested that the *A. diazotrophicus* population is sensitive to the plant genotype. They observed that only in one of their cultivars the *A. diazotrophicus* population increased during the time of the study (15 months), but in the other ones, they did not detected any trend in the bacterial numbers.

The effect observed on the *A. diazotrophicus* population colonizing sugarcane does not seem to be a direct negative effect of the fertilizer on the bacteria. We did not detect negative effects on wild-type and $GusA^+$ *A. diazotrophicus* strains growing in culture media supplied with high nitrogen levels (10 mM NO_3^-), and at the same nitrogen concentration the $GusA^+$ construct also expressed β-glucuronidase activity. Thus, it is more probable that the physiological state of the plant is altered by the nitrogen, and this subsequently affects its association with the endophyte. Pelaez Abellan et al. [26] observed that sucrose synthesis is reduced in sugarcane leaves by application of NO_3^- in a highly productive variety and increased sucrose synthesis in a variety with low productivity. In complete sugarcane plants, high nitrate doses were associated with a decrease in the concentrations of sucrose in the leaves and of the reducing sugars and sucrose in the stem [27].

A. diazotrophicus is commonly found in roughly the same numbers in sugarcane roots, stems and leaves under field conditions [6,28,29]. In the present

study, the inoculated strains were recovered from stem and root samples, and we also detected GUS activity in the stems, but not in roots, in spite of the similar bacterial numbers recovered from both organs. We cannot explain the lack of activity in root tissue, especially considering the similar numbers of *A. diazotrophicus* cells inhabiting this organ relative to the numbers inside the stem.

In this study, the expression of GUS activity was only used as a qualitative reporter of the location of the inoculated strain. Nevertheless, the lack of GUS activity in NaN_3 treated samples suggests that the *A. diazotrophicus* population in the sugarcane was lower than the limit of detection of the assay that we previously determined in vitro (2×10^5 CFU per cm^3 of tissue). That population-size indicator supported the data that was obtained by MPN technique. This estimation does not consider that X-Gluc diffusion could be limited by the plant cell walls. Another possibility is that a low proportion of bacterial cells retain the plasmid, but this does not seem to be the case, since we found plasmid maintenance to be higher than 95% after 7 months inside the plant (not shown). The absence of a bacterial growth inhibitor (NaN_3) in the histochemical incubations probably allowed an increase in the *A. diazotrophicus* population and the detection of GUS activity. Low population densities of this bacterium in sugarcane are expected as there is no evidence of specialized plant structures which harbor high concentrations of bacteria ([13–15], and this work). It is probable that *A. diazotrophicus* is almost equally distributed inside several structures throughout the bulk of the plant, and that population growth is limited, for some unknown reason, in the sucrose-rich tissues. Higher GUS activity was observed in stem xylem vessels, phloem sieve tubes and cortex, and by scanning microscopy *A. diazotrophicus*-like rods were found in cells of the stem cortex, adhering to the inner cell wall. We presumed those cells to be *A. diazotrophicus* because of their physical similarity to cells grown in culture, and since they were found only in *A. diazotrophicus* inoculated plants and were more abundant in GUS positive sections. We do not know if the plant cells seemingly colonized by *A. diazotrophicus* were damaged or alive. By immunogold labeling, James et al. [13] detected *A. diazotrophicus* inside cells from the cortex of sugarcane plantlet roots, and inside the xylem vessels from the base of the stem. They suggested that the root xylem could be the route for stem and leaf xylem infection. Our work supports previous results from another group [13,15] in that the cavities formed by the xylem secondary wall are one of the preferentially colonized microhabitats. Dong et al. [14] have proposed that *A. diazotrophicus* is found in the intercellular spaces of the stem storage parenchyma, where there are plentiful nutrients [30]. James et al. [13] also suggested that the stem xylem and the leaf xylem might be the final colonized environments inside the cane. In addition, we present evidence that *A. diazotrophicus* could colonize the stalk cortical tissue as well as its xylem. From the xylem, the bacterium might preferentially migrate to the cells of selected tissues, such as the cortex. This ultimate distribution may provide a more favorable environment for the endophyte and its N_2-fixing activity, considering that the xylem apoplastic fluid is almost devoid of carbon sources [31]. Some pathogenic and mildly pathogenic bacteria of sugarcane also colonize and survive in the xylem elements, and from there translocate to other places [32,33]. In another work, Dong et al. [34] claimed that the xylem vessels were an improbable colonization site for *A. diazotrophicus*, since after introducing this bacterium inside the stem, the plant reacted by producing substances that may have clogged the vessels. Nevertheless, their experiment probably did not reflect the natural association between *A. diazotrophicus* and sugarcane, since they made their observations on plants that were recently stressed by wounds. Moreover, as they inoculated stems by submerging their cut ends for several days in a growing bacterial suspension, with the consequent release of metabolic products, the defense reaction observed might have been expected with any bacteria.

Dong et al. [34] asserted that the xylem vessels of sugarcane were discontinuous, preventing the transport of *A. diazotrophicus* through the xylem. We presume that even if the xylem vessels are limited in their ability to translocate particulate material, *A. diazotrophicus*, and probably other species adapted to this environment, could induce plant morphological changes, such as formation of continuous vessels, by releasing plant growth regulators. It has been previously shown that *A. diazotrophicus* produces

auxins in a minimal culture medium [10]. The hypothesis of the role of *A. diazotrophicus*, is based on the observation that during the course of xylem element formation, the walls that separate adjacent vessel cells are hydrolyzed in a process that seems to be controlled by the presence of auxins [35].

The promoter used for expression of *gusA* is known to be active in eukaryotic tissues. Nevertheless, a DNA fragment that includes a duplicated CaMV promoter plus a leader sequence of AMV (alfalfa mosaic virus) was shown to induce high β-glucuronidase activity under different conditions in *A. diazotrophicus*. The bacterial recognition of eukaryotic promoter sequences might not be entirely surprising as it is known that some plant plastid promoters share consensus sequences with −35 −10 bacterial promoters [36]. Moreover, at least one eukaryotic transcription factor (TFIID) is known to show high similarity with bacterial σ-factors [37]. Particularly, the most similar region between TFIID and the σ-factors has been suggested to interact with DNA, binding to the eukaryotic TATA box in TFIID, or to single stranded DNA and to −10 bacterial promoters, in the bacterial factors. In addition, the presence of a leader sequence could enhance the translation of the *gusA* transcript, as has been observed with mRNA in different Gram-negative bacteria [38].

Under the conditions used here, sugarcane plants up to 8 month of age showed no differences in development when inoculated with *A. diazotrophicus*. From preliminary results in our laboratory, no nitrogenase expression was detected in planta from an *A. diazotrophicus* strain containing a *nifH–gusA* fusion, nevertheless we do not discard the possibility of beneficial effects of the bacteria in plants grown under other conditions, as have been reported by Sevilla et al. [39]. *Azoarcus* sp., another endophytic diazotroph has also been located inside root cortical cells of Kallar grass and inoculated rice [40]. In this association, the authors found some beneficial effect on biomass and protein content in rice plants inoculated with this bacterium.

Endophytic relationships are becoming an interesting field for studying plant-bacteria interactions and their study is still at an initial phase. Two threats to the naturally occurring endophytic associations are the high N-fertilization levels used in the modern agriculture, and the now common use of tissue culture to propagate pathogen-free sugarcane. Both practices will probably eliminate diazotrophic bacteria, as reported in this work, and sugarcane producers should be aware of this situation.

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