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Original article

Colonization of sugarcane plantlets by mixed inoculations with diazotrophic bacteria

A.L.M. Oliveira[a,d], M. Stoffels[c,e], M. Schmid[c], V.M. Reis[b,*], J.I. Baldani[b], A. Hartmann[c]

[a]Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[b]Embrapa Agrobiologia, Seropédica, Rio de Janeiro, RJ, Brazil
[c]Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Department Microbe-Plant Interactions, Neuherberg/Munich, Germany

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ABSTRACT

Micropropagated sugarcane plants have been used in Brazil for almost three decades. Besides the improvement in plant health, micropropagated sugarcane carries no endophytic plant growth-promoting bacteria. The Brazilian inoculation technology to reintroduce diazotrophic bacteria in micropropagated sugarcane plantlets revealed a synergistic-like effect in PGP-bacteria mixed inoculations. The infection model of single diazotrophic bacteria species in sugarcane was studied in detail, but still many questions remain open. In this study we used a combined fluorescence *in situ* hybridization (FISH) and a cultivation based approach (MPN) to evaluate the colonization of sugarcane plantlets by mixed inocula. The highest colonization for three out of the five species studied was obtained with a mixed inoculum, when the *Azospirillum amazonense* showed an increase by almost 100 times in colonization and *Herbaspirillum* spp. and *Burkholderia tropica* was determined at 10^7 cells per gram root fresh weight. All of the inoculated bacterial species could be detected using the FISH probes 12 h after bacterial inoculation. The FISH results confirmed the MPN counts and showed differences in the population numbers and colonization behavior of particular bacterial inoculum strains in the different mixed inocula. A putative antagonistic effect among the inoculated *H. seropedicae* and *H. rubrisubalbicans* strains was observed using FISH, as well as the better competitiveness of *B. tropica* as compared to the *A. amazonense* strain. The observed data probably reflect also specific interactions with the sugarcane variety used in this particular inoculation system, and may not be generalized as a rule. This is the first study about the competition for sugarcane colonization in a mixed bacterial inoculum.

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* *Corresponding author*. Embrapa Agrobiologia, BR 465, km 07, 23851-970 - Seropédica, Rio de Janeiro, Brazil. Tel. + 55 21 3441 1566; fax + 55 21 2682 1230.
E-mail address: veronica@cnpab.embrapa.br (V.M. Reis).
[d] Present address: Universidade Estadual de Londrina, Departamento de Bioquímica e Biotecnologia, PR.
[e] Present address: University of Applied Sciences Weihenstephan, Freising, Germany.
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1. Introduction

Axenic sugarcane meristem cultures have been adopted commercially in Brazil since the late 80's [10], aiming to finally improve plant health in sugarcane fields. Nevertheless, the sugarcane micropropagation technique suffers from the elimination of natural diversity of beneficial-pathogenic endophytic bacteria interaction [30]. The potential use of diazotrophic plant growth -promoting bacteria for the improvement of sugarcane performance has been demonstrated using greenhouse experiments as well as under field growth conditions [13,19,21,22,26]. Additional plant growth-promoting (PGP) effects like the protection against pathogens and environmental stresses [23,28] and the stimulation of root development by rhizobacterial phytohormone production [6,7,17] can be put into practice using inoculations with multiple PGP-bacterial inocula for improving the sustainability of agricultural production.

The inoculation technology developed to reintroduce diazotrophic bacteria in micropropagated sugarcane plantlets [30] has been used successfully in field trials, although the detailed spatial-temporal dynamics of colonization of sugar cane plants by diazotrophic bacterial populations are not known in detail. Recent studies about growth-promoting effects in sugar cane induced by inoculations with bacterial mixtures revealed positive and negative bacteria–bacteria interactions, plant–bacteria interactions, and plant–bacteria–environment interactions [26]. It was suggested that the inoculated bacteria are actively influenced by the plant genotype, cropping conditions and by coinoculated or residing bacterial populations which can considerably influence the resulting PGP-effects. Until now, no particular endophytic bacterial species were definitively proven to be responsible for improving neither the nitrogen supply (through BNF) nor other growth-promoting effect in sugarcane [17].

Concerning the use of a single diazotrophic bacterial species as PGP-bacterium in sugarcane, the infection model in micropropagated sugarcane was studied in detail [16]. However, the colonization and infection pattern following multiple species inoculation in micropropagated sugarcane plants has not yet been described mainly due to the difficulty to detect specifically a particular bacterial species within a mixed inoculum. Regarding to this, the fluorescence *in situ* hybridization (FISH) methodology has been used successfully to trace inoculated bacterial species, e. g. in roots of wheat [3,31] and to evaluate the community structure of bacterial populations in complex environments as bulk soil and rhizosphere soil [34,37]. To our knowledge, no study about the competition for colonization in a mixed bacterial inoculum has been reported in sugar cane by a combined FISH and cultivation based approach yet. In this work, we aimed to study the early infection and colonization of micropropagated sugarcane plantlets inoculated with different mixtures of diazotrophic PGP-bacterial species, using the FISH technique, to specifically evaluate the spatial and temporal behavior of inoculated PGP-bacteria species in a controlled system for sugar cane inoculation used in Brazil.

2. Materials and methods

2.1. Biological material and plant inoculation

Rooted micropropagated sugarcane variety SP70-1143 was kindly provided by Centro de Tecnologia da Cana (http://www.ctc.com.br, older Copersucar), and treated as previously described [26]. Briefly, the inoculants were prepared by growing each strain overnight in 5 mL of Dyg's liquid medium. Samples were counted in a Neubauer chamber and normalized to 10^9 cells mL^{-1} using sterile Dyg's medium. The inoculation mixtures were obtained by mixing equal volumes of each normalized inoculum, to reach the bacterial mixtures as presented in Table 1. Flasks of 250 mL capacity containing 50 mL of modified MS medium for sugarcane inoculation were prepared according to Reis et al. (1999). Four to six rooted plantlets were inoculated with 0.1 mL bacterial mixture (resulting in a density of 2.0×10^6 cells mL^{-1} for each strain) in the modified MS. After inoculation, the plants were maintained in a growth chamber at 30 °C and 12 h photoperiod (50 μmol m^{-2} s^{-1} of active photosynthetic light). Mixed PGP-bacterial inocula used are described in Table 1. The Embrapa Agrobiologia Culture Collection (BR 465-RJ, km 47 – CP 74.505, CEP 23.890-000 – Seropédica, RJ, Brazil) gently provided the five bacterial species: a *Gluconacetobacter diazotrophicus* BR 11281

Table 1 – Bacterial strains, isolation sources, mixtures of inoculation used in this study and respective N-free semi-solid media used for MPN counts

Bacterial species and code used	Strain	Isolation source	N-free semi-solid media
Gluconacetobacter diazotrophicus (Gd)	BR 11281 (PAL5)	Roots – *Saccharum* sp. (hybrid)	LGI-P
Herbaspirillum seropedicae (Hs)	BR 11335 (HRC54)	Roots – *Saccharum* sp. (SP70–1143)	NFB
Herbaspirillum rubrisubalbicans (Hr)	BR 11504 (HCC103)	Stems – *Saccharum* sp. (SP70–1284)	NFB
Azospirillum amazonense (Aa)	BR 11115 (CBAmC)	Stems – *Saccharum* sp. (CB45–3)	LGI
Burkholderia tropica (Bk)	BR 11366 (Ppe8)	Buds – *Saccharum* sp. (SP71–1406)	JMV
Mixture 1 – Gd			LGI-P
Mixture 2 – Hs + Hr			NFB
Mixture 3 – Bk + Aa			JMV + LGI
Mixture 4 – Gd + Hs + Hr			LGI-P + NFB
Mixture 5 – Gd + Hs + Hr + Aa + Bk			LGI-P + NFB + LGI + JMV

strain, a *Herbaspirillum seropedicae* BR 11335 strain, a *H. rubrisubalbicans* BR 11504 strain, an *Azospirillum amazonense* BR 11115 strain and a *Burkholderia tropica* BR 11366 strain. Three replicates were tested for each treatment.

2.2. Most probable number (MPN) counts

For the determination of MPN counts, samples were taken 5 days (120 h) after bacterial inoculation. The roots were surface sterilized, homogenized, serially diluted, inoculated in selective semi-solid N-free medium and identified as previously described [25]. The quantification was achieved using the McCrady table (Table 1).

2.3. Sampling and fixation for fluorescent in situ hybridization (FISH)

Plant samples for FISH-analysis were sequentially collected after 6, 12, 24, 48 and 72 h after bacterial inoculation. At each sampling time, the plantlets were removed from the inoculation flasks and washed three times in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.2) under shaking of 200 rpm for 5 min each time. After the washings, the roots were cut in sections of 2–5 mm and fixed in 2 ml of fixation solution (4% paraformaldehyde in PBS) for 12 h at 6 °C. After fixation, the roots were washed twice for 5 min in PBS, and stored frozen (−20 °C) in an Ethanol:PBS solution (1:1, v/v) until their use for FISH. Control bacterial mixtures were fixed in 4% PFA and kept in Ethanol:PBS as described above until FISH-analysis.

2.4. Fluorescent in situ hybridization

Oligonucleotide probes were purchased from MWG-Biotech (Ebersberg, Germany). Fixed roots were dehydrated by serial incubation for 3 min each in 50%, 80% and 100% thanol. Dehydrated roots were placed on gelatine-coated slides (0.075% gelatin-0.01% $CrK(SO_4)_2$). Fluorescence *in situ* hybridization was performed at 46 °C for 3 h, by adding 13 μl of hybridization solution (50 ng of each oligonucleotide probe, 0.9 M NaCl, 20 mM Tris–HCl pH 8.0, 0.01% SDS) to each root sample, using the hybridization and washing stringencies recommended for each probe or probe set (Table 2). Excess of probes were removed by washing the roots in washing solution (20 mM Tris–HCl pH 8.0; 0.1% SDS; 5 mM EDTA pH 8.0) for 20 min at 48 °C, and salt excess was removed by dipping the roots in water. DNA-staining with 4,6-diamidino-2-phenylindole (DAPI) was applied after the FISH-procedure, using 20 μl drop of a 1 μg/ml DAPI solution on each root sample, incubated on ice in the dark for 10 min. Finally, the samples were rinsed quickly with distilled water; air dried, mounted on citifluor antifading reagent, and then was immediately observed with a Zeiss Confocal Laser Scanning Microscope (LSM 510 Axiovert 100 M).

3. Results

3.1. Quantification of bacterial colonization using MPN-method

The bacteria were quantified using the MPN-method and selective nitrogen-free semi-solid media 5 days (120 h) after inoculation. The highest colonization density, after applying surface disinfection with chloramine T, was observed for *G. diazotrophicus* (higher than 1.4×10^7 bacteria per cells g^{-1} root fresh weight) when applied alone, as well as for *B. tropica* and *Herbaspirillum* spp. when inoculated in mixtures 2– 5 (Table 3). When *G. diazotrophicus* were applied in mixtures, its colonization densities were about 10 times lower as compared to its single inoculation treatment. In contrast, the *Herbaspirillum* spp. did not show decreases in its colonization efficiency due to single or mixture inoculation, as well as *B. tropica*, which was observed in high cell densities in both mixed inoculations tested. The *Herbaspirillum* endophytic populations were up to 10^7 cells g^{-1} root fresh weight when evaluated 120 h after inoculation (Table 3). A distinction between *H. seropedicae* and *H. rubrisubalbicans* bacterial species in semi-solid medium was

Table 2 – Oligonucleotide probes used in this study to detect the bacterial cells in mixed inoculations

Probe name	Specificity	Position/stringency	Reference
EUB-338-I, II, III	Eubacteria	16S, 338–355/0–50	[2]
ALF-1B	Subclass alpha-*Proteobacteria*	16S, 19–35/20	[18]
SUBU-1237	*Burkholderia* and *Suttorella*	16S, 1237–1254/60	[36]
AZO-440a + AZO-440b	*Azospirillum* genus	16S, 440–457/50	[36]
Herb-1432	*Herbaspirillum* genus	16S, 1432–1449/35	[32]
Hrubri-445	*H. rubrisubalbicans*	16S, 445–462/60	[32]
Hsero-445	*H. seropedicae*	16S, 445–462/35	[32]
Adia	*Gluconacetobacter diazotrophicus*	23S, 49–66/55	[14]

Inoculated bacterial species	Probe combinations	Stringency
Gluconacetobacter diazotrophicus	EUB-338-Fluos, ALF-1B-Cy3 and Adia-Cy5	40
Herbaspirillum rubrisubalbicans and *H. seropedicae*	EUB-338-Fluos, Hsero-445-Cy3 and Hrubri-445Cy5	35
Burkholderia tropica and *Azospirillum amazonense*	EUB-338-Fluos, SUBU-1237-Cy5, and AZO-440a + b-Cy3	50
G. diazotrophicus, *H. rubrisubalbicans* and *H. seropedicae*	ALF-1B-Fluos, Hrubri-445-Cy5, and Hsero-445-Cy3	35
G. diazotrophicus, *H. rubrisubalbicans*, *H. seropedicae*, *Burkholderia tropica* and *A. amazonense*	EUB-338-Fluos, Adia-Cy3, and HERB-1432-Cy5, or EUB-338-Fluos, SUBU-1237-Cy5, and AZO-440a + b-Cy3	40 40

Table 3 – Endophytic population of diazotrophic bacteria 120 h after inoculation

Inoculum composition	Endophytic population (log cell number g^{-1} fresh weight)			
	G. diazotrophicus	*Herbaspirillum*	*A. amazonense*	*B. tropica*
Mixture 1	>7.15	ND	ND	ND
Mixture 2	ND	>7.15	ND	ND
Mixture 3	ND	ND	4.65 ± 0.82	>7.15
Mixture 4	6.20 ± 0.32	>7.15	ND	ND
Mixture 5	6.32 ± 0.57	>7.15	6.98 ± 0.45	>7.15

Values are means of three replicates. ND – not determined.

not possible. On the other hand, an increase by almost 100 times in the *A. amazonense* colonization density was observed when inoculated in the most complex mixture as compared to its inoculation together with *B. tropica*. Even more, the MPN-counts showed a much higher population of *B. tropica* in relation to *A. amazonense* (about 1000 times higher) in the inoculation of mixture 3 (Table 3).

3.2. *FISH-analysis with controlled mixtures of bacteria using a multiple probe approach*

Fig. 1A– C demonstrate the specific *in situ* identification and detection of fixed bacterial cells using the FISH approach with multiple probes with different hierarchy and fluorescent label. Applying the three oligonucleotide probes EUB-338-I, II, III-Fluos, ALF-1B-Cy3 and Adia-Cy5 simultaneously (as indicated in Table 2), a white image results in the case of fixed *G. diazotrophicus* cells (mixture 1) after overlaying all three color channels, because all three probes are binding simultaneously (Fig. 1A). When the three probes EUB-338-I, II, III-Fluos, Hsero-445-Cy3 and Hrubri-445-Cy5 are applied on fixed cells of mixture 2 (*H. seropedicae* and *H. rubrisubalbicans*, Fig. 1B), *H. seropedicae* cells are stained yellow, because the Fluos- and Cy3-labelled probes were binding both (mixed color of green and red in the rgb-color circle, see color circle in Fig. 1A). *Herbaspirillum rubrisubalbicans* cells were labeled turcoise, as resulting staining after overlay of the green and blue channel. As further example, results of FISH-analysis of fixed cells of mixture 4 (*G. diazotrophicus*, *H. rubrisubalbicans* and *H. seropedicae*) was given in Fig. 1C. Using the probes ALF-1B-Fluos, Hsero-445-Cy3 and Hrubri-445-Cy5 simultaneously *G. diazotrophicus* cells are stained green, since it is an alpha-proteobacterium, while *H. seropedicae* and *H. rubrisubalbicans* are beta-proteobacteria. These are stained specifically with the species-specific oligonucleotide probes with a red or a blue label, respectively (Fig. 1C).

3.3. **In situ** *detection of bacteria in sugar cane plantlets using DAPI- and FISH-analyses*

DAPI staining could effectively be used to identify the bacterial colonization sites on sugarcane roots in a general way, demonstrating that bacterial cells colonize mainly the root caps and intercellular spaces of the rhizodermis (data not shown). All inoculated bacterial strains could be detected using the FISH approach with rRNA-targeted oligonucleotide probes 12 h after inoculation, albeit at different colonization densities. All inoculation mixtures analyzed 72 h after inoculation showed a biofilm formation of attached cells covering completely the root surface. These cells gave no FISH signal but were detectable by DAPI staining (data not shown). The FISH-results generally confirmed the MPN-counts, suggesting differences in the population numbers of bacterial strains inoculated in the respective combinations, as compared to single inoculations. The results of the FISH-colonization patterns determined with each inoculum combination are presented in the following in detail.

The results of FISH-analysis in sugarcane plantlets inoculated with mixture 1 showed that *G. diazotrophicus* cells were generally difficult to be determined by FISH-analysis *in planta*. Applying ALF-1B as single probe, weak hybridization signals were observed, indicating that the bacterial cells had rather low metabolic activity, or the *G. diazotrophicus* cells were difficult to be stained by FISH when not in pure culture for unknown reasons (data not shown). We found the first detectable hybridization signals in cells attached to the root epidermis 24 h after inoculation.

After inoculation with mixture 2 (*H. seropedicae* and *H. rubrisubalbicans*), both *Herbaspirillum* species could be specifically identified using the fluorescently-labeled oligonucleotide probes Hsero-445-Cy3 and Hrubri-445-Cy5 (Table 2, Fig. 1B). *H. seropedicae* could be detected already 6 h after inoculation to be firmly attached to the root surface. The hybridization signals of the probe Hsero-445-Cy3 were far more frequent than the signals derived form the probe Hrubri-445-Cy5 (data not shown). The root cap and the emergence zones of the secondary roots were the predominant colonization sites in addition to the observation that *Herbaspirillum* cells colonize effectively the rhizodermis at the late sampling times (Fig. 1E,F). An interesting observation was the absence of common colonization sites shared by the two *Herbaspirillum* species.

The first positive signals for plantlets inoculated with the inoculation mixture 3 (*Burkholderia tropica* and *Azospirillum amazonense*) were specific to *B. tropica* attached to the root cap (Fig. 1D). *Burkholderia tropica* cells were clearly found predominant as compared to *A. amazonense* cells. The *A. amazonense* cells could be identified attached to the root surface and root cap only 24 h after inoculation (data not shown). In contrast to the colonization behavior of the two *Herbaspirillum* species, *B. tropica* and *A. amazonense* cells could be specifically identified colonizing the same sites (Fig. 1D) mainly at the root cap.

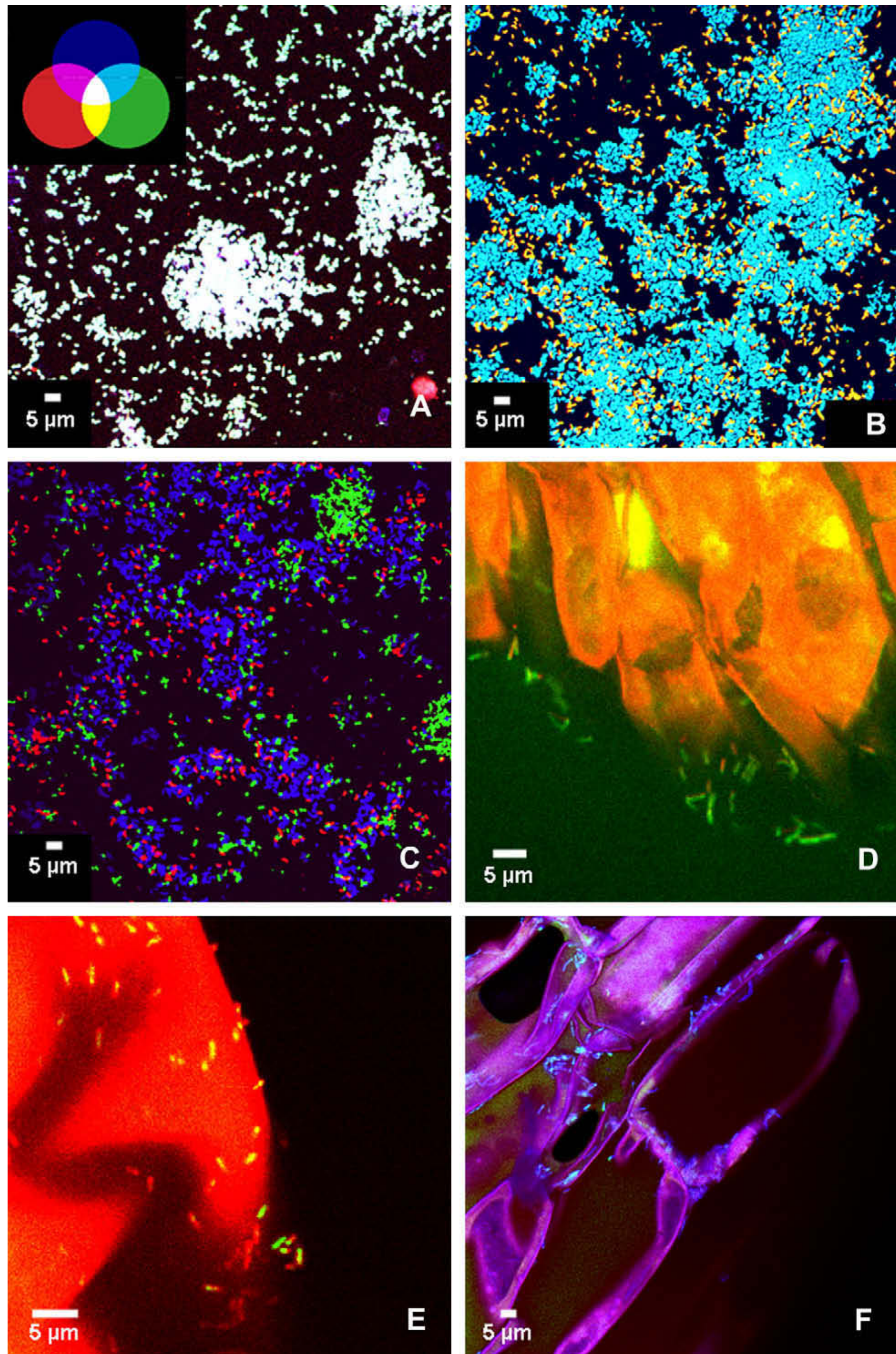

Fig. 1 – Laser scanning microscopic images of fluorescence *in situ* hybridization (FISH). Analysis of mixtures of bacteria (controls) (A – C) and of bacteria associated to roots of sugarcane micropropagated plantlets (D – F). A: Mixture 1 (*G. diazotrophicus*) stained with EUB-338-I, II, III-Fluos, ALF-1B-Cy3, and Adia-Cy5; B: mixture 2 (*H. seropedicae* and *H. rubrisubalbicans*) stained with EUB-338-I, II, III-Fluos, Hsero-445-Cy3 and Hrubri-445-Cy5; C: mixture 4 (*G. diazotrophicus*, *H. seropedicae* and *H. rubrisubalbicans*) stained with ALF-1B-Fluos, Hrubri-445-Cy5, and Herso-445-Cy3. Inoculation treatments were performed according to Table 1 and probes and labels used according to Table 2. The scale bars indicate 10 μm. (D) FISH detection of *B. tropica* and *A. amazonense* using the probes EUB-338-I, II, III-Fluos, SUBU-1237-Cy5 and AZO-440a + b-Cy3 colonizing the root cap 24 h after inoculation (inoculation mixture 3). (E) FISH- detection of *G. diazotrophicus* and *H. seropedicae* using the probes ALF-1B-Fluos, Hrubri-445-Cy5, and Herso-445-Cy3 colonizing the root epidermis 72 h after inoculation (inoculation mixture 4); (F) FISH detection of *H. seropedicae* using the probes EUB-338-I, II, III-Fluos, Adia-Cy3 and HERB-Cy5 colonizing the root epidermis 24 h after inoculation (inoculation mixture 5).

Applying inoculation mixture 4 (*G. diazotrophicus*, *H. rubrisubalbicans* and *H. seropedicae*), *H. seropedicae* cells could be identified by the species-specific probe Hsero-455-Cy3 on the root surface of inoculated plantlets (red labeled cells, Fig. 1E). *Gluconacetobacter diazotrophicus* cells could be detected with the probe ALF-1B-Fluos (green labeled cells or yellow labeled cells because of the overlay with the red fluorescence in the background derived from the plant tissue). The *H. seropedicae* strain predominates over the *H. rubrisubalbicans* strain, as observed by the fluorescent signals and agreed with the results of inoculation with mixture 2. The most prominent attachment sites were the root cap, emerging zone of secondary roots, and in a lower frequency the rhizodermis. Again, the samples collected 72 h after inoculation rendered no FISH signal and presented a biofilm of attached cells.

The FISH-analysis of plants inoculated with mixture 5 (*G. diazotrophicus*, *H. seropedicae*, *H. rubrisubalbicans*, *Burkholderia tropica* and *A. amazonense*) showed the predominance of root colonization by *Herbaspirillum* cells (double staining with the probes EUB-338-Fluos and HERB-1432-Cy5, resulting in turquoise staining) over *Gluconacetobacter diazotrophicus* (detected with probe Adia-Cy3), but no yellow stained bacteria were visible (Fig. 1F). The *Herbaspirillum* cells were attached to the root surface and were detected 6 h after inoculation, attached to the rhizodermis, and its specific hybridization signal was predominantly observed in the samples collected 12, 24 and 48 h after inoculation. Specific hybridization signals of *B. tropica* and *A. amazonense* cells were also detected (data not shown), but in a lower frequency than the *Herbaspirillum* signals.

4. Discussion

Fluorescent *in situ* hybridization (FISH) analysis based on rRNA targeted oligonucleotide probes allows the differentiation and quantification of complex bacterial communities without cultivation [1,9,20,35]. However, it is limited to cells with high ribosome content which usually are most active. Plant-associated bacteria have already been studied successfully using FISH, including one study with sugarcane [12]. Using a combination of FISH and epifluorescence detection with a strain-specific monoclonal antibody, the colonization efficiency and competitivity of two *A. brasilense* strains colonizing wheat roots were investigated [4].

Here, the FISH technique was applied to evaluate the colonization of micropropagated sugarcane plantlets by inoculated bacteria using an inoculation system that has been used in sugarcane growth-promotion experiments [26]. The results observed in this work for mixed bacterial inoculations confirms that all the inoculated species reached the endophytic habitat of micropropagated sugarcane plantlets through active infection of the root cap and emerging zone of secondary roots, although with different efficiencies due to apparently different competitiveness for colonization. Information regarding the spatial and temporal dynamics of sugarcane colonization by mixed diazotrophic bacterial inocula are provided by this communication for the first time regarding the *in vitro* sugarcane inoculation system used in Brazil [25].

The absence of hybridization signals derived from the applied Adia probe could reflect a problem of probe access to the target site in the single *G. diazotrophicus* inoculation, rather than a diminished metabolic activity. This effect was previously reported [3] for a probe targeted *A. brasilense* rRNA. In this study, the *G. diazotrophicus* Pal5 reached population densities higher than 10^7 cells per plantlet 5 days after inoculation if inoculated alone, but its population decreased about 10 times in a mixed inoculation. *G. diazotrophicus* was able to colonize the same sugarcane tissue together with other diazotrophic species, like *H. seropedicae*. The colonization sites recorded for *G. diazotrophicus* using FISH probes are in agreement with previous studies [15,30].

A putative antagonistic effect among the inoculated *H. seropedicae* and *H. rubrisubalbicans* strains was observed, suggesting that the used *H. seropedicae* strain outcompete the *H. rubrisubalbicans* strain. In respect to the time course of root colonization by *H. seropedicae* and *H. rubrisubalbicans*, the detection of *H. seropedicae* as soon as 6 h after inoculation, presents this species as one of the most aggressive in colonizing sugarcane plantlets. Olivares et al. [24] studied the colonization of sugarcane plantlets by *H. seropedicae* and *H. rubrisubalbicans* in single inoculations in detail using a similar *in vitro* inoculation system, reporting endophytic populations up to 10^7 per gram of root fresh weight and up to 10^9 cells g^{-1} aerial parts fresh weight. They also reported a complete colonization of the entire root axis in a random pattern by both *Herbaspirillum* spp. already 24 h after inoculation using scanning electronic microscope (SEM). These findings contrasts with our results, but could be explained by differences in the microscopic techniques and the vigorous root washing step used in this study. Indeed, our study showed for the first time the colonization of the root surface by active cells, followed by the selective colonization of the root cap and the zone of secondary root emergence, where the bacterial cells formed microcolonies, in agreement with Olivares et al. [24]. The interaction between the two *Herbaspirillum* species in inoculum mixture 2 suggests that *H. seropedicae* HRC54 is more competitive than *H. rubrisubalbicans* HCC103 at *in vitro* inoculation conditions. Since *H. rubrisubalbicans* is a potential pathogen in some susceptible sugar cane varieties causing the mottled stripe disease, this characteristic should also be related to the colonization pattern observed in our study which used a resistant sugarcane variety.

Azospirillum is by far the most studied PGP-bacteria [6,11], including sugarcane [8,16,29]. Sugarcane colonization by *B. tropica* is been only little studied with only two records to our knowledge [13,27]. In this work, the FISH and MPN results of the inoculum mixture 3, which comprises the *A. amazonense* and *B. tropica*, suggests that *B. tropica* has higher competitiveness and colonization efficiency at the given *in vitro* inoculation conditions. The observed attachment sites at the surface of the sugarcane plantlets roots are in agreement with the infection sites previously reported for *Azospirillum* in other grasses such as the root cap, the secondary roots emergence zone and intercellular spaces in the rhizodermis.

The sugarcane inoculation with the mixture 5 inoculum, containing all five PGP-bacterial species, provided particularly interesting results of FISH images and MPN counts. The population levels observed with this inoculum combination were

the highest observed for *A. amazonense*. The bacterial cell density of inoculum 5 increased faster than in the other inoculum mixtures, and could indicate a synergistic effect of the inoculated species in the mixture. Experiments reviewed by Bashan and Holguin [6] pointed out that a cocultivation of *Azospirillum* spp. with other bacteria promoted significantly its nitrogenase activity.

Bacteria of the genus *Herbaspirillum*, essentially *H. seropedicae*, are potentially the most efficient species in the early colonization of roots of sugarcane plantlets at this *in vitro* system. Sugarcane plants of SP70-1143 variety inoculated with a mixture of *Herbaspirillum* (*H. seropedicae* and *H. rubrisubalbicans*) showed a higher population of *H. seropedicae* attached to the roots; both species were able to colonize the root system, but at different sites indicating some antagonism. The inoculation of sugarcane plantlets with a mixture of five diazotrophic species points to a putative synergistic effect favoring the colonization by *A. amazonense*.

The antagonistic interaction effects observed among the *Herbaspirillum* species, as well as the better competitiveness of *B. tropica* as compared to the *A. amazonense* species, could also be due to a better adaptation to the conditions of the *in vitro* sugarcane inoculation, than to a real competitive effect. The observed data reflect only specific interactions with the sugarcane variety used in this particular inoculation system, and may not be a general rule. Specific interactions of microbial strains with the plant genotype have been shown for sugarcane and other grasses [5,24,33]. While the sugarcane plants were completely dependent on the MS culture medium for the supply with minerals and carbohydrates, the bacterial strains which are better adapted to the specific nutritional conditions of the MS-medium constituents (at 10% strength) should have a competitive advantage. The FISH technique was suitable to track specific bacterial species during the early colonization of sugarcane plantlets in mixed inocula of different bacterial species. Thus it proofed as a helpful tool in near future to define the best composition of promising PGP-bacteria inocula for sugarcane and other grasses.

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