

# Biotechnological Potential of Endophytic Bacteria to Improve the Micropropagated Seedling of Variety RB92579 Sugarcane (*Saccharum officinarum* L.)

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## Abstract

Endophytic bacteria may influence agricultural production in several ways, including promoting plant growth. Two experiments were conducted in order to evaluate the combination of endophytic bacteria from the Brazilian Northeast region aims at the commercial introduction of the inoculation of these bacteria in micropropagated sugarcane plants using a temporary immersion bioreactor. One experiment was done in tubes with sterile commercial substrate, and the other was done in pots with soil; both were installed in a greenhouse. A mixed inoculation was performed in six inoculated endophytic diazotrophic bacteria in micropropagated sugarcane plants, variety RB92579. In the experiment with soil, the mixed inoculation significantly increased the shoot dry matter of plants without the addition of nitrogen fertilizer. However, the accumulation of total-N in the tissues showed no significant differences between treatments with and without nitrogen fertilization. The evaluation of micropropagated seedlings showed no increases in the parameters tested. The results showed that the response of inoculation in temporary immersion bioreactor micropropagation is possible, and that the application of homologous strains may have contributed to a better response by the interaction of endophytic bacteria with sugarcane RB92579. Further studies should be conducted to improve the methodology, which indicates a great potential to optimize this process on a commercial scale.

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## Keywords

Biological Nitrogen Fixation, Meristem Culture, Diazotrophic Bacteria, Endophytes, Micropropagation

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## 1. Introduction

Brazil is the largest producer of sugarcane, with a planted area of approximately 8.6 million hectares, with an estimated production for the 2018/19 season of 62,596 million tons [1]. The production is concentrated in the South-Central and Northeast regions. This culture demands a high amount of nitrogen, the most limiting macronutrient for crop productivity. It is one of the highest costs for farmers. Since fertilizer is not subsidized in Brazil, most commercially used genotypes were chosen aiming to obtain a high productivity with low levels of soil N, favoring; even indirectly, the selection of varieties that are capable of covering part of the need for N by the association with diazotrophic bacteria [2].

The biological process of converting dinitrogen to ammonia is called nitrogen fixation (BNF), and is performed exclusively by the enzyme nitrogenase, so that it is a great support for the increase in productivity. Moreover, it is also an ecological and more economical alternative [3]. Research has shown that the key to the success of BNF processes lies in the selection of diazotrophic bacteria that can associate more efficiently. Therefore, a more detailed study on the community of diazotrophic bacteria during plant growth cycles is necessary. Studies on inoculation of micropropagated sugarcane plants with a mixture of five strains from different species showed contributions of around 30% [4]. Nitrogen fixation has a profound agronomic, economic, and ecological impact owing to the fact that the availability of fixed nitrogen represents the factor that most frequently limits agricultural production throughout the world [5].

Micropropagation is a practice widely used in many countries in Europe, Asia, United States and Brazil. This method is based on the production of more uniform and healthy plants and on a much higher growth speed within a limited physical space [6]. However, endophytic microorganisms have been mentioned in several studies as contamination sources to micropropagation. Others consider their presence as a positive factor, arguing that they are able to assist plants, since they live inside their tissues without causing symptoms of their presence, and in the case of *in vitro* cultivation they can favor osmotic adjustment, production of phytohormones and absorption of nutrients [7] [8]. The reintroduction of diazotrophic endophytic bacteria in micropropagated sugarcane plants has helped studies on the association between plants and diazotrophic bacteria, allowing us to evaluate the potential of BNF and growth promotion [4] [9]. In this context, the present study was performed to evaluate the micropropagated sugarcane seedlings using a temporary immersion bioreactor system aiming the

commercial introduction of bacterial inoculation as well as the consequent benefit for the culture through a BNF process and/or other mechanisms for promoting plant growth.

## 2. Material and Methods

### 2.1. Culture Media

The culture media used for isolation of bacteria were LGI-P, JNFb, NFb and JMV according to [10]. The medium DYGS [11] was used for the growth of strains and for DNA extraction.

### 2.2. Isolation and Quantification of Bacteria

Triplicate samples of roots and stems of the sugarcane variety RB92579 were obtained from a field in the state of Paraíba (06°57'25.6877"S latitude and 35°07'06.1412"W longitude), Brazil. The culms were disinfected with a pre-wash of the surface using soap and water, and then scrubbed with cotton-soaked 70% alcohol. The roots were disinfected with 70% alcohol for 30 seconds, then washed with sodium hypochlorite (2.5%) for 1 minute under agitation and with sterile water for five times during 5 minutes. After disinfestation, the roots were ground in 90 mL of a saline solution, thus characterizing a dilution of  $10^{-1}$ , with three replications [10]. Then, they were diluted serially in 0.1 mL of suspension and inoculated in vials containing 5 mL of semisolid free-N, LGI-P, NFb, JNFb and JMV. According to Dobreiner *et al.* [10], each medium is selective for a particular genus of diazotrophic bacteria: NFb (*Azospirillum* spp), JNFb (*Herbaspirillum* spp), LGI-P (*Gluconacetobacter diazotrophicus*) and JMV (*Burkholderia* spp). After 72 - 96 h of incubation, the pots with a white film on the surface were also replicated for a new source medium.

### 2.3. DNA Extraction

DNA bacterial extraction was conducted using phenol-chloroform. Endophytic bacterium isolates were grown in 5 mL DYGS [11] for 24 h at 28°C. A 400 µL aliquot of the solution was transferred to a microtube and 400 µL saturated phenol solution was added. The mixture was shaken in a vortex apparatus and subjected to centrifugation at 16,000 g for 5 min. The supernatant (aqueous layer) was transferred to a new microtube and the phenolic step was repeated. After centrifugation the supernatant was again transferred to a new microtube and 400 µL chloroform was added. The microtube was shaken in a vortex and centrifuged for 5 min at 16,000 g. The aqueous layer was transferred to another microtube, to which 1 mL cold ethanol was added. To complete the process of extracting the DNA, the microtube was centrifuged for 3 min at 16,000 g, the ethanol discarded, and the tubes incubated at 37°C for 30 min to evaporate residual solvent. The extracted material was resuspended in 15 µL Mili-Q sterile water.

## 2.4. Sequencing of Gene 16S rDNA and Gyrase $\beta$

The 16S rDNA gene was amplified as per using universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') e rD1 (5'-AAGGAGGTGATCCAGCC-3') [12]. Gyrase  $\beta$  gene was amplified using gyrB3F (5'-TCCGGCGGTCTGCACGGCGT-3') e gyrB14R (5'-TTGTCGGGTTGTACTCGTC-3') gene and PCR product for the isolates was sequenced in both directions.

PCR reactions (25  $\mu$ L) contained 10 $\times$  PCR reaction buffer, 10 mM dNTPs, 50 mM MgCl<sub>2</sub>, 10 pmol primer, 15 ng DNA and 2.5 units *Taq* DNA-polymerase (Invitrogen). The temperature profile consisted of 5 min initial denaturation at 95°C followed by 30 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 2 min followed by a final extension at 72°C for 5 min. PCR product was purified using PureLink PCR Purification Kit (Invitrogen) according to the instructions. Sequencing was performed on an ABI PRISM 9700 capillary sequencer using the ABI Prism Big Dye Terminator Cycle sequencing kit (Applied Biosystems).

The 16S rDNA and Gyrase  $\beta$  gene sequences were compared with the GenBank database (<http://www.ncbi.nlm.nih.gov/>) using BLAST. For local alignment, was used to BLASTn tool (NCBI-[www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and Multiple alignments were performed with CLUSTAL W [13]. A phylogenetic tree was constructed using MEGA 6 (version 6) [14].

## 2.5. Micropropagation of Sugarcane

Endophytic diazotrophic bacteria were used in the mixture to inoculate sugarcane plants (Table 1).

The experiment was conducted at the Northeast Strategic Technologies Center (CETENE), Recife/PE state, Brazil. Micropropagated sugarcane plants, commercial variety RB92579, were used to evaluate the effects of inoculation of diazotrophic bacteria. Micropropagated plants in a Temporary Immersion Bioreactor (TIB) that did not present contamination at the rooting phase received a mixed bacterial inoculum. For the inoculum, bacteria were grown for 48 hours

**Table 1.** Bacteria used for mixing the inoculant with the isolated plant tissue of sugarcane variety RB 92579.

Genus of bacteria	Identification number of each species.	Plant tissue
<i>Enterobacter</i>	1 b	Culm
<i>Enterobacter</i>	5	Root
<i>Enterobacter</i>	10	Root
<i>Enterobacter</i>	25	Root
<i>Gluconacetobacter</i>	30	Culm
<i>Enterobacter/Pantoea</i>	22	Root

in a liquid medium under agitation in a Dygs medium [10], at 30°C. After mixing each inoculum in equal parts, an aliquot of 6.7 mL of mixed inoculum with an optical density (O.D. 540 nm = 0.05), containing approximately  $10^4$  cells/mL, was added to TIB pots with a capacity of 5000 mL, containing 2000 mL of MS medium modified by [15] in micropropagated seedlings in rooting phase.

Two experiments were conducted at the CETENE greenhouse. One used plastic tubes filled with the sterilized commercial substrate Basaplant® and the other used pots (8 L) with non-sterile forest soil under greenhouse conditions.

In the first experiment, the mixed inoculation was evaluated with the following treatments:

- One level of inoculation: A combination of previously identified diazotrophic bacteria: *Enterobacter* + *Gluconacetobacter diazotrophicus*.
- Two levels of nitrogen fertilizer: Recommended dose ( $80 \text{ kg of N ha}^{-1}$ ), and without nitrogen fertilization.
- One non-inoculated control with nitrogen fertilization, following the recommended dose.

The experimental design was randomized blocks with four replications. The plants were kept in plastic tubes (acclimatized) for 45 days and fertilized with a nutrient solution. The effects of inoculation considering fertilization was evaluated by determining the accumulation of shoot and root dry mass. The plants were taken to the laboratory, the aerial part was cut and measured with a ruler and the constant dry mass of the samples were obtained after drying the plant material at a temperature of about 70°C (degrees Celsius) for at least 48 h. After drying, the material was weighed cold.

For the second experiment, the remainder of the seedlings that did not undergo evaluation during the first experiment was transplanted into 8-liter pots containing non-sterile soil. From this soil, several single samples were collected to form a composite sample, which was then analyzed (Table 2), obtaining the fertilization recommendation. The experimental design was randomized blocks with four replications. After 120 days after planting, evaluations of accumulation of root and shoot dry mass and the determination of total nitrogen accumulated in plant tissues were made using the Kjeldahl method [16].

## 2.6. Statistical Analysis

Each variable studied was subjected to analysis of variance (ANOVA), F test, and Tukey's test, at 5% significance levels using the statistical software ASSISTAT version 7.7 [17].

**Table 2.** Chemical characteristics of the soil sample used in the conduction of the second experiment (pots).

mg/dm <sup>3</sup>					pH	cmol <sub>c</sub> /dm <sup>3</sup>							
Fe	Cu	Zn	Mn	p		K	Na	Al	Ca	Mg	H	S.B	CTC
44.20	0.60	8.30	7.10	5	6.3	0.10	0.06	0.0	4.50	0.90	2.60	5.55	8.15

### 3. Results and Discussion

#### 3.1. Isolation of Diazotrophic Bacteria

Populations of nitrogen-fixing bacteria were higher in samples of roots compared with samples of culms. Previously conducted tests indicated that the bacteria used in this experiment have an ability to fix  $N_2$  *in vitro*, which becomes a potential tool for the production of IAA and for inorganic phosphate solubilization (**Figure 1**).

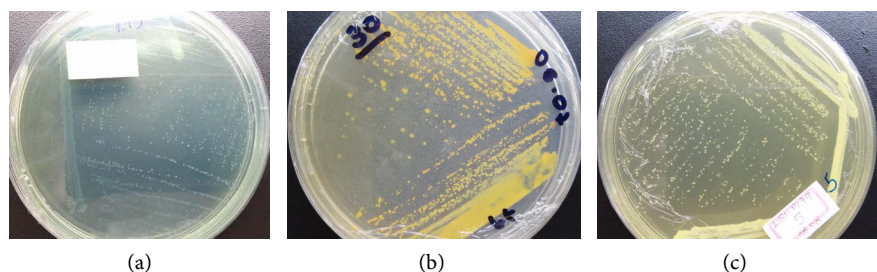
#### 3.2. Molecular Phylogeny of Bacterial Isolates

The total DNA of the six bacterial isolates was purified and used as a model for PCR (polymerase chain reaction) in order to amplify their 16S rRNA genes. The sequencing of the 16S rRNA gene allows an accurate identification of the genera of endophytic bacteria from various species of plants, including sugarcane, corn, rice and medicinal plants [18] [19] [20]. However, it is necessary to analyze other genes, such as *gyrA* and *gyrB*, to obtain a precise definition of the species and even subspecies [21]. Using a molecular approach, the occurrence of phylogenetic types of organisms and their distribution in natural communities may be studied directly from the environment. Since 1988, when only twelve phyla of bacteria were reported, the number of phyla increased due to cultivation activities, especially by the research on rRNA genes on the environment. Currently, over 70 phyla of bacteria are recorded in public databases [22]. The sequencing of rRNA genes is an efficient method of choice for phylogenetic reconstruction based on the detection of nucleic acid and the quantification of microbial diversity.

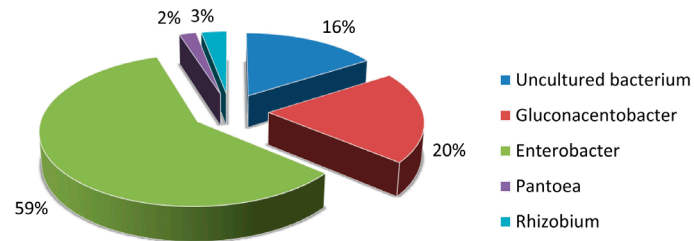
Based on the sequence of the 16S rRNA gene, the endophytic bacterial isolates in this study were identified as *Enterobacteria*, *Gluconacetobacter*, *Rhizobium*, *Pantoea* and non-cultivable bacteria (**Figure 2**).

The results of phylogenetic analyses allowed grouping the endophytic sugarcane isolates into two groups, with similarities with sequences in the GenBank public database ranging from 99% to 100% (**Figure 3**).

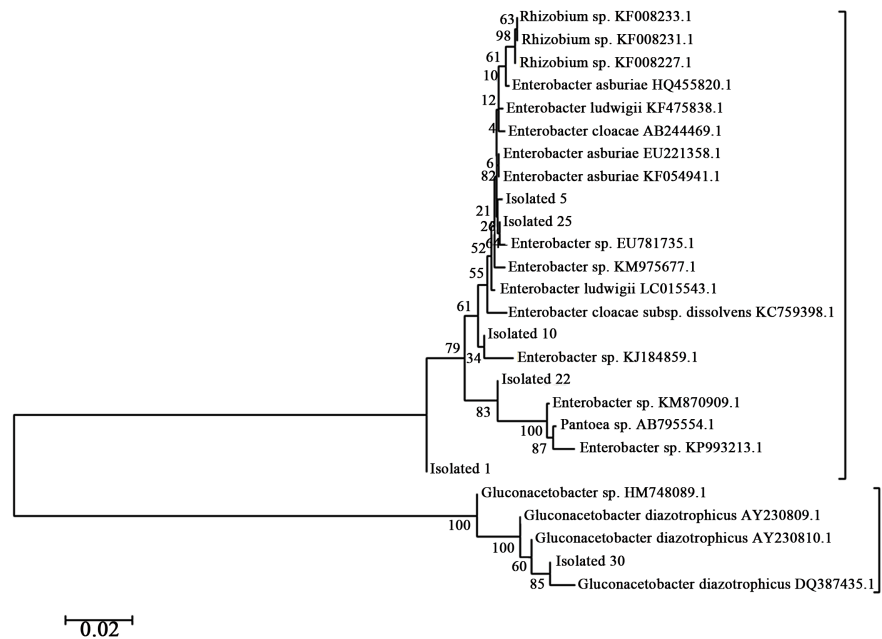
Group I was composed by the isolates 1, 5, 10, 22 and 25, which are related to *Pantoea* sp., *Rhizobium* sp., *Enterobacter* sp., *E. asburiae*, *E. ludwigii* and *E. cloacae*. This result was confirmed by the sequencing of the gene *gyrB*, which enabled identifying the isolates at the species level, such as *E. cloacae* (**Table 3**).



**Figure 1.** Colonies of diazotrophic bacteria isolated in different semi-selective medium: NFb (A), LGI-P (B), JMV (C).



**Figure 2.** Abundance of each genus identified among endophytic bacterial isolates from RB 92579 sugarcane cultivar.



**Figure 3.** Phylogenetic tree constructed with sequences of the 16S rRNA regions of endophytic bacteria isolated from sugarcane and sequences from GenBank (indicated by accession number), using the neighbor-joining method and utilizing Tamura-Nei for nucleotides, with the pairwise gap deletion option. Numbers indicate frequency of each branch from bootstrap analyses of 10,000 replicates.

**Table 3.** Isolated bacterial endophytes identified with relationship to species by sequencing of the *gyrβ* gene and the identity percentage found in the National Center for Biotechnology Information database.

Isolates	Species	Query value	E value	Identity	Accession
1	<i>Enterobacter cloacae</i>	100%	0.0	97%	AB972391.1
5	<i>Enterobacter cloacae</i>	99%	0.0	94%	AB084016.1
10	<i>Enterobacter cloacae</i>	100%	0.0	98%	AB972391.1
22	<i>Enterobacter cloacae</i>	100%	0.0	97%	AB972391.1
25	<i>Enterobacter cloacae</i>	100%	0.0	94%	AB084013.1

*Pantoea* was found in sugarcane and soybeans [23] is at soja [24]. Studies have shown the potential of *Pantoea* sp. to induce a systemic resistance and protection against pathogenic microorganisms. Additionally, these bacteria may in-



duce the growth of plants, increasing the supply of nitrogen in non-symbiotic associations, solubilizing phosphorus and stimulating the production of phytochromes [25].

Although Rhizobia infect naturally legumes as host plants, some strains may form symbiotic relations with non-legume species. Besides fixing N<sub>2</sub>, they are also capable of contributing to the promotion of growth of these species.

Group II included the isolated 30, which is related to *Gluconacetobacter diazotrophicus*. *G. diazotrophicus* is considered the main diazotrophic endophyte in sugarcane and has been isolated from leaves, stems and roots of sugarcane plants and other economically important grasses [23]. Several studies have shown that such endophytes colonize their hosts in vast numbers and cause an increase in production [26] [27]. The possibility of replacing fertilized nitrogen for biological nitrogen fixation is a very important economic and environmental factor [28].

The results obtained in this study are essential to provide the necessary knowledge on the analysis of endophytic bacteria in micropropagated sugarcane plants and to indicate the potential for future applications of endophytes that promote plant growth.

In addition to the common ability to fix N<sub>2</sub>, associative and endophytic bacteria are genetically diverse. They were identified among various genera: alpha, beta and gamma-proteobacteria, including *Azospirillum*, *Azorhizobium*, *Azoarcus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Rhizobium* [23] [29].

### 3.3. Evaluation of Micropropagated Sugarcane Seedlings

The results of this experiment showed no significant statistical differences by Tukey test ( $p < 0.05$ ) among treatments inoculated with the mixture of diazotrophic bacteria (with and without nitrogen fertilization) and the control. There was a tendency for increase for the control treatment at this micropropagated phase (Table 4).

**Table 4.** Effect of inoculation of bacteria from micropropagated sugarcane plants (RB 92579) at the 45<sup>th</sup> day.

Treatment	Height (cm)	RDM (g)	SDM (g)
Control-Nitrogen Fertilization	70.05 a	0.16 a	0.54 a
Inoculant-Nitrogen Fertilization	64.45 ab	0.09 b	0.37 b
Inoculant-Fertilization without Nitrogen	61.85 b	0.12 ab	0.43 ab
M ± SD	65.45 ± 5.92	0.12 ± 0.05	0.44 ± 0.12
CV%	11.84	32.00	37.30

RDM = root dry matter; SDM = shoot dry matter. Means followed by the same letters do not differ by Tukey test ( $p \leq 0.05$ ). M ± SD = mean ± standard deviation.



Results similar to those obtained in this study were found by [9] upon analyzing the effect of inoculation by determining the accumulation of root and shoot dry mass of sugarcane seedlings, variety SP701143, at the 65<sup>th</sup> day. In this study, no statistically significant differences were observed among treatments inoculated with 44 strains of diazotrophic bacteria and the non-inoculated control, suggesting that at this stage the plant does not respond adequately to inoculation.

### 3.4. Evaluation of Sugarcane in Pots

The height of plants in pots showed no significant differences by Tukey test ( $p < 0.05$ ) among treatments inoculated with the mixture of diazotrophic bacteria (with and without nitrogen fertilization) and the control. The addition of nitrogen fertilizer to the inoculated treatment resulted in a slight decrease in RDM (14.14 g). It did not differ statistically from the control treatment with the addition of nitrogen fertilizer (16.38 g), evidencing that N is a limiting factor in this experiment (Table 5). However, the inoculation without nitrogen fertilizer promoted a greater accumulation of root dry matter compared to the control treatment.

The evaluation of the SDM accumulation showed that the mixed inoculation promoted a positive effect on plant development without the addition of nitrogen fertilizer (41.7 g), differing from the other treatments (Table 4).

The results suggest that the inoculation used with the commercial variety RB92579 affected the interaction with inoculated bacteria. A better understanding of the plant-bacteria interaction, the selection of diazotrophic endophyte strains and the variety of cane needs to be further studied aiming a maximum benefit of BNF.

Microbial inoculants are an alternative method to increase crop productivity and may reduce the use of chemical fertilizers, which is one of the agricultural practices that affect the environment [30]. The positive effects of inoculating some bacteria during plant growth may be associated to the BNF process and the synthesis of growth hormones produced by bacteria. Among the effects associated

**Table 5.** Effect of inoculation of bacteria from micropropagated sugarcane plants (RB 92579) in pots (at the 120<sup>th</sup> day).

Treatment	Height (cm)	RDM (g)	SDM (g)	Leaf N (g/Kg dry wt <sup>-1</sup> )
Control-Nitrogen Fertilization	1.76 a	16.38 ab	35.28 b	10.39 a
Inoculant-Nitrogen Fertilization	1.76 a	14.14 b	31.71 b	9.90 a
Inoculant-Fertilization without Nitrogen	1.82 a	17.77 a	41.70 a	10.08 a
M ± SD	1.78 ± 0.10	16.09 ± 3.14	36.23 ± 5.63	10.98 ± 2.68
CV%	7.73	25.57	20.35	17.81

RDM = root dry matter; SDM = shoot dry matter. Means followed by the same letters do not differ by Tukey test ( $p \leq 0.05$ ). M ± SD = mean ± standard deviation.

with this synthesis of hormones, the growth of lateral and adventitious roots, the stimulus to cell division and the elongation of roots and stems are mentioned [31]. This may explain the accumulation of root dry matter.

Similar results were observed by some authors. Lin *et al.* [32], upon inoculating two strains of *Enterobacter* spp., observed that both strains increased the biomass content and the nitrogen of micropropagated sugarcane seedlings grown with a nitrogen fertilizer equivalent to 180 kg of urea ha<sup>-1</sup>, a recommended nitrogen fertilization dose for the cultivation of the sugarcane ROC22 at the seedling stage. [4], upon inoculating in micropropagated sugarcane plants different species of diazotrophic bacteria, isolated and in mixtures, observed that *Herbaspirillum* sp., *A. amazonense* and a combination of five strains of different bacteria showed a significant increase in the accumulation of fresh mass in the culms of plants, evidencing a 30% contribution of BNF. However, the individual inoculation of *G. diazotrophicus* promoted a negative effect on the accumulation of fresh mass of culms compared to the non-inoculated control. The results presented by [9] showed that the inoculation response in micropropagated SP 701143 seedlings at the rooting stage showed variations that may have been dependent on several factors, including plant genotype and the environment. In this study, the inoculation with the strains PAL3 and CBAmC caused a significant increase in the accumulation of culm dry matter compared to the non-inoculated control treatment. However, the accumulation of N in plant tissues grown in pots after 180 days of growth showed that plants inoculated with a mixture of the strains PAL5 and HCC103 and the individual strains HRC54, Z94 and CBAmC showed a higher nitrogen content in the tissues. [33], studying potted micropropagated seedlings, observed that the total biomass increased due to inoculation with one strain or a combination of strains without nitrogen fertilization. [34] inoculated a mixture of diazotrophic bacteria and mycorrhizal fungi in micropropagated sugarcane plants and obtained an effect equivalent to half the recommended dose of nitrogen fertilizers for potted plants. [4] demonstrated that the combined inoculation of associative and endophytic bacteria promotes a synergistic effect compared to the individual inoculation of bacteria in micropropagated sugarcane plants. Increases of 30% in the accumulation of N in plants via BNF were observed for these plants.

### 3.5. Effects on Growth

The contribution of endophytic bacteria for the nutrition of legume plants by BNF is well known. Among non-legume species, BNF is still subject of much discussion. The contributions observed are varied and depend on specific interactions among bacterial and plant genotypes [35].

The inoculation of micropropagated sugarcane plants has already been performed and resulted in interesting effects on the outcome of plants. Preliminary inoculation experiments with *G. diazotrophicus* in micropropagated sugarcane plants showed increases of up to 28% in shoot fresh matter. Promising results were also obtained when micropropagated sugarcane plants were inoculated

with the strain PAL-5 associated with small doses of nitrogen, as shown by [36].

When the colonization of the plant is established, one result arising from the association is the promotion of plant growth by direct and indirect mechanisms. In addition to fixing N, endophytic diazotrophic bacteria produce plant growth hormones such as auxin and gibberellic acid [37]. Improvements in nutrient absorption are also reported [38].

Several experiments demonstrated that endophytic bacteria may indirectly benefit the development of the plant, increasing the plant's tolerance to biotic and abiotic stresses [39] [40] [41]. Beneficial results from such associations in sugarcane plants include a significant increase in plant height and biomass, root length and production of dry matter [42] [43].

Current evidence indicates that the BNF process performed by diazotrophic bacteria may contribute up to 60% to the sugarcane's N uptake [2], and that it depends on the plant genotype and on its interaction with various associative bacteria genera [44].

Quantitative analyses of BNF and the promotion of plant growth evidenced that plant and bacterial genotypes are important factors to the control of association efficiency [45]. In this context, the determination of the best combination between diazotrophic bacteria and plant varieties to obtain the maximum benefit of such association in agriculture is a challenge in this area.

#### 4. Conclusion

This study showed that inoculation using a temporary immersion bioreactor is possible. This is the first inoculation report for seedlings using this system. The use of homologous strains may also have contributed to the benefit of the interaction with the plant (sugarcane variety RB92579). The results suggest a high response potential to inoculation and optimization of the process on a commercial scale.

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#### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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