Australasian Plant Pathology (2021) 50:513–522
https://doi.org/10.1007/s13313-021-00813-0

ORIGINAL PAPER

Plant growth promotion of micropropagated sugarcane seedlings var. Co 412 inoculated with endophytic diazotrophic bacteria and effects on the Ratoon Stunting Disease

J. B. Carneiro Jr.[1] · L. M. Barroso[1] · F. L. Olivares[2] · E. C. Ponte[1] · S. F. Silveira[1]

Received: 14 December 2020 / Accepted: 14 July 2021 / Published online: 27 July 2021

Abstract
The present study aimed to evaluate the influence of *Gluconacetobacter diazotrophicus* (PAL5), *Herbaspirillum seropedicae* (HRC54) and *Herbaspirillum rubrisubalbicans* (HCC103) on seedling vigor, plant-crop productivity and potential to control the sugarcane Ratoon Stunting Disease (RSD) caused by *Leifsonia xyli* subsp. *xyli* (Lxx). Micropropagated seedlings (variety Co 421) were inoculated with endophytic diazotrophic bacteria and transplanted to the nursery. Lxx was inoculated in seedlings 80 days before field planting (seven months) by pruning the basal leaves with scissors pre-immersed in contaminated sap. To estimate diazotrophic bacteria's population density, shoot and root samples of seedlings transplanted to the nursery were collected at 42 and 86 days after inoculation. Agronomic characteristics of sugarcane plants were evaluated at harvest (16 months). In general, there was a population reduction of the bacterial endophytes after 86 days, compared to 42 days after inoculation. *G. diazotrophicus* provided greater gains in tons of cane per hectare (TCH) (68.6%) and tons of Brix per hectare (59.1%) than the other treatments. Plants inoculated with diazotrophic bacteria and challenged with the pathogen (Lxx) showed a high incidence of Lxx seropositive in the stalks. However, an increase in yield of 18.83 and 19.09 TCH was noticed, respectively, related to control and Lxx inoculated treatment in the first harvest (or growing season). The treatment with *H. rubrisubalbicans* alone showed a low incidence of Lxx and positive agronomic yield performance, suggesting some effect on the xylem pathogen colonization for the variety Co 421.

Keywords *Saccharum* sp. · *Gluconacetobacter diazotrophicus* · *Herbaspirillum seropedicae* · *Herbaspirillum rubrisubalbicans* · Bioproducts

Introduction

Brazil is the world's largest sugarcane producer, having harvested 665.1 million tons of sugarcane in the 2020/21 harvest. The Southeast country's main producing region reached 436 million tons harvested, remarkably in São Paulo and Minas Gerais states. Its appreciation is associated with the biofuels sector, in which its main by-products are total ethanol (29.8 billion liters), anhydrous ethanol, hydrated ethanol, and sugar (41.8 million tons) (CONAB 2020).

Sugarcane crop had shown a decline of productivity affected by the increase in harvest cycles associated with abiotic and biotic factors, including the enhanced incidence of systemic pathogens, emphasizing the bacterium *Leifsonia xyli* subsp. *xyli* (Lxx)—the causal agent of ratoon stunting disease (RSD) (Davis et al. 1980, 1984; Evtushenko et al. 2000). The Lxx bacterium is fastidious, gram-positive, coryneform, without flagella, aerobic and pleomorphic. Cultivated in vitro, it produces tiny and translucent colonies after 14 days of cultivation in Soybean Corn medium (Gillaspie et al. 1981; Cardoso 1986). The disease has no characteristic symptoms; the bacterium colonizes the xylem of the plant, obstructing the translocation of water and nutrients, especially under conditions of water restriction (Hughes and Steindl 1956; Davis and Dean 1984). Quecine et al. (2016)

✉ S. F. Silveira
silvaldo@uenf.br

[1] Laboratório de Entomologia E Fitopatologia, Centro de Ciências E Tecnologias Agropecuárias, Universidade Estadual Do Norte Fluminense Darcy Ribeiro, Campos Do Goytacazes/RJ 28013-602, Brazil

[2] Laboratório de Biologia Celular E Tecidual, Centro de Biociências E Biotecnologia, Universidade Estadual Do Norte Fluminense Darcy , Campos Do Goytacazes/RJ 28013-602, Brazil

demonstrated that colonization of Lxx also occurs in mesophyll cells and cells in the vascular bundle's sheath. With successive harvests in susceptible varieties, we observe a delayed growth of cane sprouting, stunted stalks, and decreased production. In extreme cases, it evolves to plant death (Ricaud 1968). The symptoms are nonspecific, with cross-section stalks showing reddish-orange dots or lines in the nodal regions. They also arise with the attack of other diseases, insects, environmental factors, and mechanical damage (Gillaspie and Teakle 1989). Therefore, the diagnosis of RSD can only be confirmed through methods of detecting the presence of Lxx in the xylem sap, through serological tests (Carneiro et al. 2004; Urashima and Grachet 2012), which is a time-consuming method and does not detect low bacterial concentrations; or through molecular tests (Sun et al. 2019), which have greater sensitivity and specificity, however, are of higher costs (Wu et al. 2018) and do not detect only active colonized stalks.

RSD is spread rapidly through cutting tools during manual and mechanized cane harvesting (Damann 1992). The RSD control strategies aim to prevent the initial inoculums' entry into the production areas, especially through heat treatment of setts or bud setts (50.5 ºC/120 min or 52 ºC/30 min). However, these seedling treatments do not eliminate Lxx (Fernandes Jr. et al. 2010; Urashima and Grachet 2012; Dias et al. 2019). Another auxiliary method, micropropagation by a meristem culture (Sreenivasan and Sreenivasan 1984; Muthukumarasamy et al. 2006), also does not guarantee the total elimination of the bacteria. Already, the use of resistant/tolerant varieties comes up against the limited availability of studies that prove such mechanisms (Gagliardi and Camargo 2009; Young 2016; Fu et al. 2019), although there is genetic variability among the clones and progenitors of sugarcane sugar in Brazil. Finally, it is recommended to reduce the spread by disinfesting agricultural equipment, using bactericidal agents, or heat, which is rarely practiced in the field due to logistical issues (Urashima et al. 2020).

In this sense, biocontrol can be used as an alternative strategy to minimize the negative impacts caused by the disease. Endophytic bacteria, such as *Gluconacetobacter diazotrophicus*, can compete for the same colonization sites of the pathogen, stimulate resistance mechanisms in the host and produce bacteriocins (Arencibia et al. 2006; Blanco et al. 2010; Oliveira et al. 2018). *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *Burkholderia ambifaria* applied on potatoes and tomatoes in a greenhouse pot experiment were tested against *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Rhizoctonia solani*. The consortium was able to successfully counteract the infection of both fungal pathogens in pre-emergence (infection before germination) and post-emergence (infection after germination) (Pellegrini et al., 2020). Besides, *G. diazotrophicus*, *H. seropedicae*, *H. rubrisubalbicans*, *Azospirillum amazonense* and *Burkholderia tropica* colonize sugarcane roots, stalks and leaves and have been used as part of the bacterial consortium developed by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) and recommended as a commercial inoculant for sugarcane due to its plant growth promoting characteristics (Reis et al. 2008; Chaves et al. 2015; Dos Santos et al. 2019).

The hypothesis of RSD biocontrol by endophytic bacteria opens the perspective of disease control, aiming at endogenous reduction of the pathogen. Besides, endophyte inoculation can result in higher productivity and lower cost for producers, especially in varieties with low resistance and/or tolerance to RSD, thus justifying the investment in seedlings treated by thermotherapy, micropropagation, or pre-inoculated with diazotrophic endophytes. In this context, the objective of the present study was to evaluate, under field conditions, the vigor, productivity, and health of sugarcane micropropagated plants inoculated with the endophytic diazotrophic bacteria *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* and evaluate the potential of this strategy in controlling RSD, by decreasing the incidence of stalks seropositive for the presence of the pathogen Lxx on plant cane.

Materials and methods

Production of healthy Co 421 seedlings

The micropropagated sugarcane seedlings of the Co 421 variety were produced at the Meristem Culture Laboratory of the Dr Leonel Miranda Campus (UFRRJ), in which the setts were previously heat-treated at 50.5 ºC for 2 h and multiplied by meristem culture (Hendre et al. 1983). Part of the seedlings was transferred to glass flasks containing 50 mL of modified MS medium (diluted ten times, without the addition of hormones) (Murashige and Skoog 1962) and inoculated with endophytic bacteria, remaining in these conditions for seven days until transplantation nursery (Reis et al. 1999). The control condition was established without inoculation of the bacteria.

Multiplication and inoculation of the endophytic diazotrophic bacteria *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans*

The endophytic bacteria *Gluconacetobacter diazotrophicus* strain PAL5 (Gd) (Gillis et al. 1989), *Herbaspirillum seropedicae* strain HRC54 (Hs) (Baldani et al. 1986) and *Herbaspirillum rubrisubalbicans* strain HCC103 (Hr) (Baldani et al. 1996) were grown separately in liquid DIGS medium (Döbereiner et al. 1995) and incubated in a rotatory

shaker for 24 h, at 30 ºC, under agitation at 175 rpm, obtaining a final bacteria cell suspension of 10^8 cells.mL^{-1}. The mixed inoculum containing the three bacteria species was added to the seedlings in a modified MS medium (100 µL of inoculum/seedling/flask). After seven days of inoculation, the seedlings were transplanted in trays of 128 cells containing filter cake substrate, sugarcane bagasse, and soil (1:1:1) and kept in a screened nursery (50% shade) for 20 days. Then, the seedlings were transplanted in open-air beds for acclimatization for 200 days.

Pathogen inoculation

The bacterium *Leifsonia xyli* subsp. *xyli* (Lxx) (Carneiro et al., 2004) was inoculated into the seedlings eighty days before planting in the field. The inoculation was done by pruning the basal leaves with scissors pre-immersed in sap contaminated with the pathogen. The sap of infected stalks that resulted in a seropositive reaction for the presence of pathogen was obtained from the CB 49–260 variety, highly susceptible and proven to be infected by Lxx with at least 10^6 bacteria.mL^{-1}, according to "Dot Blot" serological sensibility (Carneiro et al. 2004). This sap was used to inoculate all the seedlings.

Planting and experimenting in the field

The experiment was installed at the Dr Leonel Miranda Campus (UFRRJ), in a randomized block design, with four replications and nine plots each, arranged in lines of 8 m, the spacing between lines of 1.40 m and between plants of 0.5 m, 2 m firing line and distal borderline. Field planting was established with seedlings of approximately seven months. During the conduct of the experiment, irrigation or fertilization was not performed. Each treatment consisted of approximately 150 seedlings of the Co 421 variety, distributed in 1 - G. diazotrophicus (Gd); 2 - H. seropedicae (Hs); 3 - H. rubrisubalbicans (Hr); 4 - L. xyli subsp. xyli (Lxx); 5 - Gd + Lxx; 6—Hs + Lxx; 7 - Hr + Lxx; 8 - Gd + Hs + Hr + Lxx; 9 -Control (micropropagated seedlings without inoculation of bacteria).

Estimation of the endophytic bacteria population associated with the plant tissue and xylem sap

At 42 and 86 days after inoculation of the endophytic bacteria, five seedlings from each treatment were collected to quantify the population of the bacteria *G. diazotrophicus*, *H. seropedicae*, and *H. rubrisubalbicans*. For this purpose, 1 g of root or 5 g of leaves and stalks were washed in running tap water and partially disinfected with alcohol-soaked cotton. The roots, leaves and stalks samples were macerated in saline and diluted in series (10^{-2} to 10^{-7}). Then, 100 µL of each dilution was applied in semi-solid medium LGI-P (*G. diazotrophicus* selective medium) and JNFb (*Herbaspirillum* spp. selective medium), in triplicate (Baldani et al., 2014). The Most Probable Number (MPN) of bacteria was estimated according to the McCrady Table, based on the absence or presence of a white surface pellicle into the glass vial containing the semi-solid medium, which represents the positive growth for diazotrophic bacteria (Döbereiner et al. 1995). To confirm the inoculated bacteria's identity, a platinum loop of the bacterial pellicle of the last positive dilution from JNFb and LGI-P was transferred to a new semi-solid medium. The new-formed pellicle was streaked in JNFb and LGI-P solid medium plates containing 20 mg.L^{-1} of yeast extract as a nitrogen source. Typical *Herbaspirillum* white colonies with greenest to the blue centre were transferred to JNFb semi-solid medium to confirm the diazotrophic ability by pellicle formation (Matteoli et al. 2020). Then, a portion of cells was harvested with a platinum loop, mounted in a glass slide with a coverslip and examined under a phase-contrast light microscope for the presence of small, curved rods that are fast-moving when near air bubbles. The species level confirmation was possible by contrasting the carbon source used by the bacterial re-isolates, where *H. seropedicae* strain HRC54 could use N-Acetyl-D-glucosamine and Myo-inositol and unable to use Meso-erythritol. *H. rubrisubalbicans* strain HCC103 showed an opposite pattern (Matteoli et al. 2020). Almost the same procedure was adopted for *G. diazotrophicus* strain PAL 5 new-formed pellicle streaked in LGI-P solid medium plates. Typical orange-coloured colonies were transferred to LGI-P semi-solid medium to confirm pellicle formation. Under a phase-contrast light microscope, *Gd* cells are small immobile rods (Baldani et al. 2014).

To detect the presence of endophytic bacteria in the xylem sap, a composite sample consisting of 10 stalks per plot was extracted at harvest after 16 months in the field. The xylem sap samples were obtained as described by Carneiro et al. (2004) and diluted from 10^{-1} to 10^{-4} in saline and applied in LGI-P and JNFb semi-solid medium in triplicate. The MPN was estimated as previously described.

Evaluation of agronomic characteristics

Thirty days after planting in the field, the percentage of seedling establishment, the number of seedlings, the height (cm), and the average diameter (cm) of tillers were evaluated. After 16 months of planting in the field, the experiment was harvested, first counting the number of stalks per plot (NSP). Then, 30 stalks in the sequence were removed from the center of each plot, which were

used for the following evaluations: average stalk diameter (cm), measuring 10 stalks per plot at the height of the fifth basal internode with a calliper; average stalk weight (ASW) (g) estimated by the relationship between the weight of 30 stalks /30; the share weight (g), estimated by multiplying the ASW and the NSP; the Brix grade, estimated with a field refractometer and sampling five older stalks of the plot, at the height of the fifth basal internode; tons of cane per hectare (TCH) estimated by calculating the useful area of the extrapolated plot to 10,000 m^2; and tons of Brix grade per hectare (TBH) estimated using the equation TCH*Brix/100.

Estimation of stalk incidence with RSD by serology

Sampling was carried out in the experimental plots, randomly removing 10 stalks per plot. Samples of xylem sap were taken from the third basal internode of the stalks for use in the "Dot Blot" serological test, as described by Carneiro et al. (2004). The mean incidence of the disease in the plot was estimated by the incidence of stalks (IS) that presented seropositive reactions for Lxx estimated by the formula: (number of positive reactions/number of samples)*100.

Statistical analysis

The results obtained from the agronomic characterization and the estimation of stalk incidence with Lxx were subjected to analysis of variance (ANOVA), followed by Scott and Knott means cluster test, at 5% probability, using the statistical program Genes (Cruz 2001). The results were presented as mean ± standard deviation. The graphs were created using the statistical program GraphPad Prism 5.0.

Results

Estimation of the endophytic diazotrophic bacteria associated with micropropagated seedlings

The MPN estimated the density of the endophytic bacteria (Table 1) in a semi-solid medium. At 42 days, *G. diazotrophicus* was higher in the root than in the aerial part in the treatments in which it was inoculated separately. *H. seropedicae* was recovered from the shoot in the Hs treatment and the root in the treatment Hs + Lxx treatment. On the other hand, *H. rubrisubalbicans* showed high density in the root in the Hr-treatment and the aerial part in the treatment combining Hr + Lxx. At 86 days, the density of *G. diazotrophicus* was also higher in the Gd-treatment, but there was a reduction in the population size related to the first sampling time for both treatments, in the root and the aerial part. There was a recovery in both tissues for *H. seropedicae* bacteria and *H. rubrisubalbicans*, regardless of inoculation with Lxx.

For the treatment that received the mixed inoculum, it was observed that the population of *G. diazotrophicus* and *Herbaspirillum* spp. were not detected in the root and that only the population of *Herbaspirillum* spp. was detected in the aerial part at 42 days. On the other hand, at 86 days, *G. diazotrophicus* and *Herbaspirillum* spp. were detected only at the root. In the first isolation, at 42 days, microorganisms were detected both in LGI-P and in JNFb in some plots inoculated only with Lxx and in the control's JNFb. In the second isolation, at 86 days, the presence of bacteria in the Lxx treatment was not quantified, and only a small amount at the root in LGI-P medium.

Table 1 Bacterial density (cells/gram of fresh biomass) in the root and shoot of sugarcane plants, at 42 and 86 days, estimated by the most probable number (MPN)

Treatment	42 Days				86 Days			
	Root		Shoot		Root		Shoot	
	LGI-P	JNFb	LGI-P	JNFb	LGI-P	JNFb	LGI-P	JNFb
Gd	2.0×10^5	-	2.3×10^4	-	9×10^2	-	1.5×10^3	-
Hs	-	0	-	2.3×10^5	-	1.5×10^3	-	1.8×10^3
Hr	-	1.5×10^6	-	1.5×10^5	-	3.5×10^3	-	1.8×10^3
Lxx	0	0	4.5×10^4	1.5×10^4	0	0	0	0
Gd + Lxx	1.5×10^5	-	2.3×10^5	-	3×10^2	-	4.5×10^2	-
Hs + Lxx	-	3×10^5	-	0	-	3×10^3	-	1.8×10^3
Hr + Lxx	-	0	-	7.5×10^5	-	1.5×10^3	-	4.5×10^2
Gd + Hs + Hr + Lxx	0	0	0	7.5×10^5	4×10^2	7.5×10^2	0	0
Control	0	0	0	4.8×10^5	4×10^2	0.0	0	0

Results expressed as "−" indicate that the culture medium is not selective for the treatment microorganism; Results expressed as "0" indicate that there was no microbial growth in selective culture medium, Gd − *G. diazotrophicus*; Hs − *H. seropedicae*; Hr − *H. rubrisubalbicans*; Lxx − *L. xyli* subsp. *xyli*

Table 2 Results of the number, height (cm) and average diameter (cm) of tillers of sugarcane seedlings inoculated with endophytic bacteria and Lxx 30 days after transplanting to the field

	Nº tillers	Height (cm)	Tiller diameter (cm)
Gd	78.75 ± 27.32 a	75.42 ± 9.14 a	13.88 ± 0.83 a
Hs	41.75 ± 22.10 a	49.62 ± 5.66 b	7.65 ± 1.73 b
Hr	50.50 ± 11.96 a	51.42 ± 4.26 b	7.47 ± 1.41 b
Lxx	66.00 ± 29.23 a	50.21 ± 3.32 b	6.50 ± 0.38 b
Gd + Lxx	39.25 ± 10.37 a	54.13 ± 4.45 b	7.56 ± 0.69 b
Hs + Lxx	74.75 ± 17.90 a	53.67 ± 5.43 b	8.20 ± 1.22 b
Hr + Lxx	52.25 ± 33.76 a	52.03 ± 7.89 b	7.53 ± 1.84 b
Gd + Hs + Hr + Lxx	60.00 ± 6.78 a	58.48 ± 3.67 b	8.17 ± 2.13 b
Control	52.25 ± 26.16 a	47.08 ± 5.11 b	7.27 ± 1.36 b
C.V	37.10	10.00	16.24
F	1.62	9.46	10.04

Averages ± standard deviation followed by the same letter are not significantly different at P<0.05; C.V. – coefficient of variation; F – ANOVA (P≤0.05); Gd – *G. diazotrophicus*; Hs – *H. seropedicae*; Hr – *H. rubrisubalbicans*; Lxx – *L. xyli* subsp. *xyli*

In isolation performed at 16 months, a low population density of endophytes was obtained from the raw xylem sap. In the Hr treatment, low populations were detected in all blocks, ranging from 0.4×10^2 to 2.5×10^2 bacterial cells/mL of raw cane sap. In a single portion of the Hr + Lxx treatment, 2.5×10^2 bacterial cells/mL was detected. In control, only in block 4, *Herbaspirillum* was detected at 9.5×10^2 bacterial cells/mL and *Gluconacetobacter* 4.5×10^2 bacterial cells/mL.

Seedling vigor after 30 days of planting in the field

After transplanting to the field, the parameters evaluated at 30 days demonstrated that the height and average diameter of the tillers per plot showed better results for seedlings inoculated with exclusively *G. diazotrophicus*, while no significant differences ($P \leq 0.05$) were found for the other treatments (Table 2).

Agronomic characteristics of sugarcane plants

Among the agronomic parameters analyzed at the end of the harvest, only the number of stalks per plot and average stalk diameter did not show significant differences ($P \leq 0.05$) between treatments (Table 3).

For the average stalk weight, the best results were obtained from the treatments: Gd, Hr, Gd + Lxx, Hs + Lxx and Hr + Lxx. As for the stalk weight per plot, the treatments that stood out were Gd, Hr + Lxx and Hs + Lxx. In both cases, the treatment Lxx and the control presented values below the average – 1.63 and 150.42 kg, respectively. Regarding the Brix grade, the treatment in which all bacteria were inoculated differed significantly from the other treatments. The worst Brix grade was obtained from the Gd treatment, below the mean of 16.67 (Table 3).

Regarding the productivity of cane-plant in tons/hectare, at 16 months, it was found that for TCH the treatments Gd, Hr + Lxx, and Hs + Lxx stood out from the others. It is noteworthy that the Gd treatment exceeded the control by 68.5% and that the treatments Hr + Lxx and Hs + Lxx were

Table 3 Results of the characteristics associated with the vigor and productivity of the sugarcane harvest at 16 months

	Average number of stalks	Average diameter of stalks (cm)	Average weight of stalks (g)	Parcel weight (g)	Brix
Gd	119 ± 11 a	2.72 ± 0.43 a	1.73 ± 0.12 a	205.96 ± 22.41 a	15.06 ± 1.67 b
Hs	75 ± 13 a	2.64 ± 0.39 a	1.52 ± 0.07 b	114.90 ± 21.74 b	16.31 ± 1.26 b
Hr	91 ± 13 a	2.80 ± 0.05 a	1.70 ± 0.05 a	153.31 ± 20.53 b	16.93 ± 0.70 b
Lxx	81 ± 21 a	2.75 ± 0.20 a	1.48 ± 0.37 b	122.02 ± 46.56 b	16.43 ± 1.35 b
Gd + Lxx	79 ± 15 a	2.80 ± 0.20 a	1.80 ± 0.22 a	143.39 ± 41.68 b	16.44 ± 0.80 b
Hs + Lxx	105 ± 15 a	2.68 ± 0.25 a	1.64 ± 0.19 a	172.27 ± 40.87 a	16.86 ± 1.41 b
Hr + Lxx	96 ± 36 a	2.85 ± 0.11 a	1.82 ± 0.23 a	176.08 ± 71.44 a	16.58 ± 0.35 b
Gd + Hs + Hr + Lxx	93 ± 22 a	2.62 ± 0.10 a	1.54 ± 0.21 b	143.65 ± 39.42 b	19.26 ± 1.00 a
Control	85 ± 31 a	2.56 ± 0.09 a	1.46 ± 0.16 b	122.19 ± 34.96 b	16.08 ± 1.59 b
C.V	20.54	9.03	11.06	22.9	7.42
F	2.16	0.61	2.26	3.02	3.27

Averages ± standard deviation followed by the same letter are not significantly different at P<0.05; C.V. – coefficient of variation; F – ANOVA (P≤0.05); Gd – *G. diazotrophicus*; Hs – *H. seropedicae*; Hr – *H.rubrisubalbicans*; Lxx – *L. xyli* subsp. *xyli*

41.2% and 44.4% higher, respectively, superior to the treatment inoculated only with the pathogenic bacterium Lxx. Such results are equivalent to 46.6 t of stalks/ha, on average (Fig. 1).

For TBH, the treatments Gd, Hr, Hr + Lxx, Hs + Lxx, Gd + Hr + Hs + Lxx are significantly higher than the others ($P \leq 0.05$), presenting higher values than the general average (22.28 t of Brix/ha) (Fig. 2). Together, these treatments exceeded 7.26 t of Brix/ha of plants inoculated with Lxx only and 8.16 t of Brix/ha of plants used as control.

As for the incidence of stalks positive for Lxx, in cane-plant, the highest incidences were observed in treatments Gd, Hs, Gd + Lxx, Hs + Lxx, Hr + Lxx, and the control. The lowest incidences were observed in the treatments Hr, Lxx, and complete inoculum. The average incidence in cane-plant, was 16.39%, with the highest incidence detected in the Hs + Lxx treatment, with 32.5% (Fig. 3). The Gd, Hs and Hr treatments showed few strong positive reactions to RSD, while Hr, Lxx, and complete inoculum treatments showed very weak reactions.

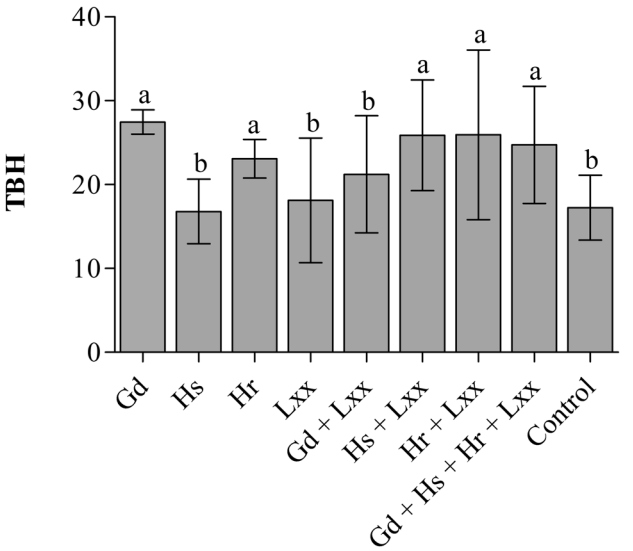

Fig. 2 Comparison of production in tons of Brix per hectare (TBH) of treatments inoculated with endophytic bacteria and with Lxx and not inoculated. Averages ± standard deviation followed by the same letter are not significantly different at $P < 0.05$. Gd—*G. diazotrophicus*; Hs—*H. seropedicae*; Hr—*H. rubrisubalbicans*; Lxx—*L. xyli* subsp. *xyli*

Discussion

The isolation procedure of endophytes from roots and shoots aimed that the bacteria inoculated have colonized the micropropagated seedlings before planting in the field, in two stages. The isolated inoculation of *G. diazotrophicus* revealed population densities greater than 10^7 cells per plant, 5 days after inoculation. However, its population has decreased about 10 times in a mixed inoculation, even though it can colonize the sugarcane tissue and other diazotrophic ones, such as *H. seropedicae* (Oliveira et al. 2009).

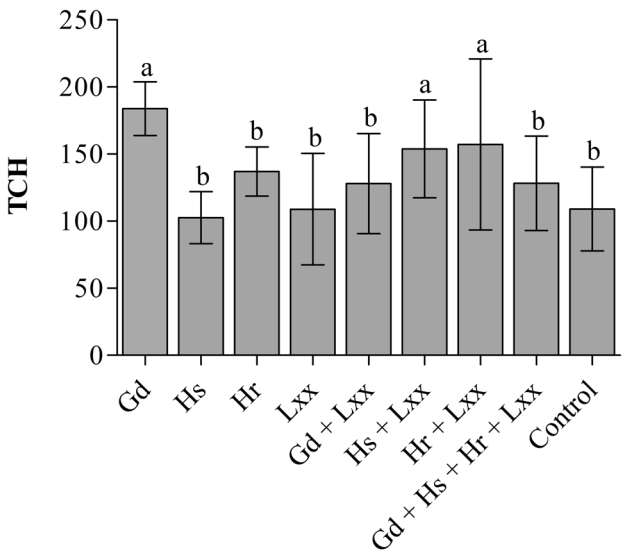

Fig. 1 Comparison of production in tons of cane per hectare (TCH), treatments inoculated with endophytic bacteria and with Lxx, and not inoculated. Averages ± standard deviation followed by the same letter are not significantly different at $P < 0.05$. Gd—*G. diazotrophicus*; Hs—*H. seropedicae*; Hr—*H. rubrisubalbicans*; Lxx—*L. xyli* subsp. *xyli*

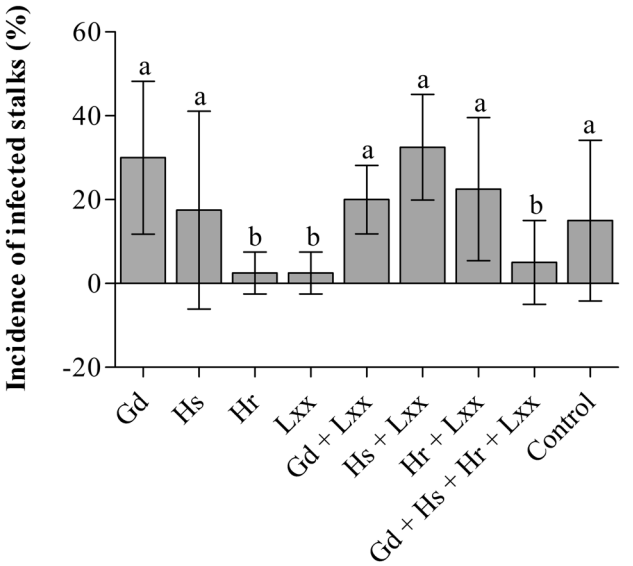

Fig. 3 Percentage of incidence of seropositive stalks for Lxx, in cane-plant, at 16 months, after transplanting micropropagated seedlings and inoculated with endophytic bacteria and Lxx and not inoculated. Averages ± standard deviation followed by the same letter are not significantly different at $P < 0.05$. Gd—*G. diazotrophicus*; Hs—*H. seropedicae*; Hr—*H. rubrisubalbicans*; Lxx—*L. xyli* subsp. *xyli*

This reduction was also observed in our results. Regarding *Herbaspirillum* spp., both species showed similar population sizes associated with the plant host; however, when inoculated together, it was not possible to identify which species was more prevalent, based on the methods that we used. In general, there was a population reduction of endophytes after 86 days, compared to 42 days after inoculation.

The detection of endophytic bacteria in the xylem sap, at 16 months, in which the stalks were mature, was reduced to a few plots, in which small densities of *Herbaspirillum* spp. and *G. diazotrophicus*, possibly because the number of endophytic bacteria in the xylem vessels, in the final stage of maturation of the sugarcane stalks, was reduced or absent. Baldani et al. (1997) mentions that *H. seropedicae* colonizes xylem vessels in small cell densities, while *H. rubrisubalbicans* colonizes the vessels in high concentrations (Olivares et al. 1997). Reis et al. (2000) reports a reduction in the *G. diazotrophicus* population size at the end of the culture cycle, from 15 months onwards, the same not being observed for *Herbaspirillum* spp. On the other hand, the pathogen population increases with maturation in the xylem vessels, with basal internodes having the greatest concentration (Harrison and Davis 1988). Thus, it was expected that the population of endophytic bacteria, in the xylem sap, at 16 months would be low. However, *H. rubrisubalbicans* in the inoculated plants showed that this bacterium also colonizes the xylem vessels in the basal internodes of sugarcane stalks and could compete with Lxx for the same colonization sites.

Regarding the important characteristics for agricultural production (number of tillers; stalk height and diameter up to 30 days; the number of stalks per plot (NSP); average stalk diameter; average stalk weight (ASW); plot weight; Brix grade; tons of cane per hectare (TCH); and tons of Brix per hectare (TBH)), a positive effect was observed mainly for *G. diazotrophicus*. When inoculated separately, the vegetative vigor induced by this bacterium led to an excessive sprouting of tillers with different maturation ages and a greater weight of the total portion, consequently causing a lower Brix degree. Even so, its inoculation produced higher TCH and TBH. The association between Gd + Lxx, on the other hand, resulted in an increase of 17.5% in HCT and 16.8% in TBH compared to treatment with Lxx alone. The high incidence of RSD in plants inoculated with Gd and Gd + Lxx demonstrated that the clonal cleaning recommended by heat treatment and meristem culture does not guarantee Lxx seedlings' cleaning/or did not prevent further reinfection. Despite this, the gains with vigor and productivity of plants inoculated with Gd, outweighed the null effects of the presence of Lxx until the time of harvest. This response is due to Gd's effects at the beginning of the plant's growth, before the increase in the population density of Lxx, which occurs with the maturation of the stalks.

The inoculation with *H. seropedicae* harmed the characteristics associated with productivity compared to plants inoculated with Lxx and control. Olivares et al. (1997) report different interaction levels between bacteria and plant species or an affinity between strains and cultivars. This fact explains, in part, the low initial development that later resulted in low agricultural yields in plants with Hs. *H. seropedicae* can also induce hypersensitivity reactions in sugarcane leaves (Olivares et al. 1997). However, in this work, the cultivar used Co 421 did not show any symptoms of mottled stripe disease, only small chlorosis in the leaves and reduced initial development in the inoculated plants. However, there is no information on disease symptoms with the inoculation of Hs or Hr in the cultivar Co 421. It was also observed that the association of Hs + Lxx promoted gains in TCH and TBH in comparison with the control and the plants inoculated with Lxx only, revealing an intriguing positive effect of the microorganism-plant interaction. The competition between bacteria (Hs and Lxx) may have stimulated the greater activity of *H. seropedicae,* benefiting the host plant through mechanisms of biological nitrogen fixation and production of phytohormones, for example (Serrato et al. 2010; Monteiro et al. 2012). Similary, Dall'Asta et al. (2019) evaluated the effects of *H. seropedicae* SmR1 strain on the maize growth and leaf anthracnose (*Colletotrichum graminicola*) of plants. Although the bacterium has efficiently colonized the leaf tissues and promoted maize growth, it did not affect the disease severity in leaves. Although Hs does not affect the colonization of Lxx, as the infected stalk incidence was high in plants inoculated with Hs and Hs + Lxx, there was better yield in the second treatment than treatment with the pathogen alone.

The performance of *H. rubrisubalbicans* in plants inoculated with Hr + Lxx was superior to that of plants inoculated with Hr only in the parameters of ASW, plot weight, and TCH, indicating a positive interaction between these bacteria, as observed for Hs + Lxx. On the other hand, unlike Hs, the isolated Hr bacterium positively affected TBH and had a lower incidence of seropositive stalks for Lxx. Hr's small densities were detected at 16 months, indicating that this bacterium was present in the xylem sap and can colonize its vessels, competing in some way with Lxx. It is noteworthy that *H. rubrisubalbicans* can establish beneficial non-pathogenic interactions with Poaceae, but it is also capable of causing disease in some susceptible sugarcane varieties (Schmidt et al. 2012), which was not observed for Co 421.

The effects of the association of Gd + Hs + Hr + Lxx bacteria on the Brix degree and TBH were positive, surpassing the plants inoculated with Lxx (17.2 and 36.2%, respectively) and the control (18.8 and 43.3%, respectively). These microorganisms (Gd, Hs, and Hr) were not isolated from the xylem sap at 16 months, and this treatment may be

correlated with the low population density of endophytic bacteria recovered during the initial isolations. On the other hand, this treatment had a low incidence of stalks positive for Lxx. Considering that greater microbial diversity is associated with a lower individual population density due to the greater competition for nutrients, space, and oxygen (Maron et al. 2011; Van Elsas et al. 2012), the endophyte bacteria, together, promoted gains in the quality of sugar cane. *G. diazotrophicus*, *H. seropedicae*, *H. rubrisubalbicans*, *A. amazonense* and *B. tropica*, both individually and in a mixture, in the absence of pathogens, promote biomass gain, increase in nutrient absorption and changes in root architecture in two sugarcane cultivars (cv. RB867515 and IACSP95-5000) (Dos Santos et al. 2019).

Plants inoculated in isolation with Lxx had low agricultural yield, being below the general average for most of the evaluated parameters (except tillers height, at 30 days and average stalk diameter, at 16 months) similar as results obtained with the control - o that was not expected. Zhu et al. (2018) obtained a reduction of 12.8% in plant height, 14% in stalk diameter and 12.1% in stalk weight, on average, after 210 days of Lxx inoculation, compared to healthy plants. The low incidence of seropositive stalks with Lxx in treatments with Lxx was surprising since the inoculation of the plants was carried out simultaneously and place and with the same inoculum as the other treatments. The hypothesis that best justifies the low incidence of Lxx in the plots inoculated with the pathogen would be the variation in the explants' health used in vitro multiplication. Despite this, the low agricultural yield associated with the low incidence of RSD indicates that even small Lxx concentrations can reduce production in a variety susceptible to RSD, such as Co 421. The micro-propagated plants not inoculated (control) showed low agricultural yield and, even high incidence of sera-positive stalks for Lxx, which confirms that thermotherapy at 50.5 ºC/120 min/sample of buds, associated with the culture of meristem does not promote the eradication of RSD bacteria on xylem or sap (Damann and Benda, 1983).

Together with the plant's morphological and physiological changes, Lxx negatively regulates the cell cycle of young plants, affects hormonal defense mechanisms and modulates the activity of antioxidant enzymes, both in varieties considered susceptible and resistant to RSD (Zhang et al. 2016; Cia et al. 2018; Fu et al. 2019; Faria et al. 2020). Plants infected with Lxx have lower indole-3-acetic acid (IAA) and gibberellic acid (GA3) content and increased abscisic acid (ABA) after 180 days of emergence (Zhang et al. 2016). Considering that the hormonal balance is also important for plant growth, the endophytes used in this study may have acted in the regulation of this balance, considering that they are capable of producing phytohormones (Monteiro et al. 2012), justifying the gains in productivity before the effects of the high incidence of seropositive stalks and suppressing the negative effects of Lxx-induced RSD.

It is estimated that the RDS reaches 25 to 85% of Brazilian crops, 10% of commercial crops in the Center-South of Brazil and that 32% of the plants monitored have been using contaminated matrices (Ponte et al., 2010; Urashima et al. 2010), representing an annual economic loss of US $ 1 million (Urashima et al. 2017). In this sense, considering that the successive cuts of the cane promote a natural decline in harvest productivity even in the absence of Lxx, the gains with the inoculation of Gd in cane-plant are justified by themselves, since we obtained a 19% increase in TCH in comparison with the control treatment and the inoculated with Lxx. Although the effect on increasing productivity is reduced in successive cane crops and the negative effect of Lxx is cumulative, Gd inoculation has a significant impact on the economy. Suppose the same responses (results) were obtained with other varieties in Brazil. In that case, we can estimate that this increase in biomass in plant cane can increase gains by $ 209.76/ha, equivalent to $ 1.8 millions per harvest (according to CONAB 2020/2021, Brazil harvested 8,605 thousand ha, productivity was 77 t/ha, and the average value of cane delivered to the field was $ 14.34 – considering the current dollar exchange rate). Studies are suggested to evaluate the plant's genotypic effect on the interactions with diazotrophic bacteria in promoting growth, suppression of symptoms, and damage induced by inoculation or infestation by Lxx in sugarcane seedlings. Additional research is also needed to evaluate the effect of these endophytes on promoting plant growth, tolerance to RSD and on the spread of the pathogen in stalks after conduction of the next crop season (ratoon cane), under representative commercial crop practices and according to the harvesting method, if manual or mechanized.

Conclusions

The bacterium *G. diazotrophicus* provided the greatest gains in tons of cane per hectare (68.6%) and tons of Brix per hectare (59.1%). In the combined treatments of endophytes with the pathogen there was an improvement in agronomic yield, in sugarcane plant with at least 18.83 t of cane/ha, in comparison with the control, and 19.09 t of cane/ha in comparison with the treatment with Lxx, despite positive serological reactions in the plots inoculated with endophytes. The treatment with *H. rubrisubalbicans* alone showed a low incidence of Lxx and good agronomic yields, suggesting some negative effect of the pathogen's colonization in the xylem of cane-plant for the variety Co 421.

Acknowledgements The authors would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro), UENF (Universidade Estadual do Norte Fluminense Darcy Ribeiro) and FENORTE (Fundação Estadual do Norte Fluminense) for the

post-graduate scholarships granted and for logistical, human and material support in the development of the research project and, UFRRJ (Universidade Federal Rural do Rio de Janeiro) for the release of the first author to make his doctoral studies at UENF.

Author contribution This manuscript is original and has not been submitted for publication in another journal. This work is part of the doctoral thesis of Doctor Josil de Barros Carneiro Junior, under the guidance of Professor Silvaldo Felipe da Silveira, as required by the Graduate Program in Plant Production at UENF, in Campos dos Goytacazes, RJ, Brazil. The first author performed most of the experimental conduct, data collection and laboratory analysis. All authors contributed to the conception and design of this research and commented on the previous versions of the manuscript and approved the final format of the manuscript for this submission to the APP.

References

Arencibia AD, Vinagre F, Estevez Y et al (2006) *Gluconoacetobacter diazotrophicus* elicitate a sugarcane defense response against a pathogenic bacteria *Xanthomonas albilineans*. Plant Signal Behav 1(5):265–273

Baldani JI, Baldani VLD, Seldin L, Döbereiner J (1986) Characterization of *Herbaspirillum seropedicae* gen. nov., a root-associated nitrogen-fixing bacterium. Int J Syst Bacteriol 36:86–93

Baldani JI, Caruso L, Baldani VLD, Goi SR, Döbereiner J (1997) Recent advances in BNF with non-legume plants. Soil Biol Biochem 29(5/6):911–922

Baldani JI, Pot B, Kirchhof G, Falsen E, Baldani VL, Olivares FL, Hoste B, Kersters K, Hartmann A, Gillis M, Dobereiner J (1996) Emended description of *Herbaspirillum*; inclusion of [*Pseudomonas*] *rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. Nov.; and classification of a Group of Clinical Isolates (EF Group 1) as *Herbaspirillum* species 3. Int J Syst Bacteriol 46(3):802–810

Baldani JI, Reis VM, Videira SS, Boddey LH, Baldani VLD (2014) The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. Plant Soil 384(1):413–431

Blanco Y, Legaz ME, Vicente C (2010) *Gluconacetobacter diazotrophicus*, a sugarcane endophyte, inhibits xanthan production by sugarcane-invading *Xanthomonas albilineans*. J Plant Interact 5(4):241–248

Cardoso CON (1986) Isolamento da bactéria do raquitismo (*Clavibacter xyli* subsp *xyli*) no Brasil. Boletim Técnico COPERSUCAR, São Paulo 34:48–52

Carneiro Jr JB, Silveira SFD, Souza Filho GAD, Olivares FL, Giglioti ÉA (2004) Especificidade de anti-soro policlonal a *Leifsonia xyli* subsp. *xyli*. Fitopatol Bras 29(6):614–619

Chaves VA, Santos SGD, Schultz N, Pereira W, Sousa JS, Monteiro RC, Reis VM (2015) Desenvolvimento inicial de duas variedades de cana-de-açúcar inoculadas com bactérias diazotróficas. Rev Bras Cienc Solo 39(6):1595–1602

Cia MC, de Carvalho G, Azevedo RA et al (2018) Novel insights into the early stages of ratoon stunting disease of sugarcane inferred from transcript and protein analysis. Phytopathol 108(12):1455–1466

CONAB. Companhia Nacional de Abastecimento. (2020) Acompanhamento da safra brasileira de cana-de-açúcar. v7 - Safra 2020/21, n. 3 - Terceiro levantamento, Brasília, p. 1–62, dezembro de 2020

Cruz CD (2001) Programa Genes (versão Windows): Aplicativo computacional em genética e estatística. Viçosa: UFV, 648 p

Dall'Asta P, Velho AC, Pereira TP, Stadnik MJ, Arisi ACM, (2019) *Herbaspirillum seropedicae* promotes maize growth but fails to control the maize leaf anthracnose. Physiol Mol Biol Plants 25(1):167–176

Damann KE (1992) Effect of sugarcane cultivar susceptibility on spread of ratoon stunting disease by the mechanical harvester. Plant Dis 76(11):1148–1149

Damann KE, Benda GTA (1983) Evaluation of commercial heat-treatment methods for control of ratoon stunting disease of sugarcane. Plant Dis 67(9):966–967

Davis MJ, Dean JL (1984) Comparison of diagnostic Aechrigues for determining incidence of ratoon stunting disease in Florida. Plant Dis 10:896–899

Davis MJ, Gillaspie AG, Harris RW, Lawson RH (1980) Ratoon stunting disease of sugarcane: isolation of the causal bacteria. Science 210:1365–1367

Davis MJ, Gillaspie AG, Vidaver AK, Harris RW (1984) *Clavibacter*, a new genus containing some phytopathogenic coryneform bacteria *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. Int J Syst Bacteriol 34:107–117

Dias VD, Carrer Filho R, Cunha MGD (2019) Comparação de detecção molecular de *Leifsonia xyli* subsp. *xyli* em toletes de cana-de-açúcar tratados termicamente. Pesq Agropec Trop 49:e55132

Döbereiner J, Baldani VLD, Baldani JI (1995) Como isolar e identificar bactérias diazotróficas de plantas não leguminosas. Brasília, EMBRAPA-SPI. Itaguaí, RJ, EMBRAPA-CNPAB, p 40

Dos Santos SG, Chaves VA, da Silva RF, Alves GC, Reis VM (2019) Rooting and growth of pre-germinated sugarcane seedlings inoculated with diazotrophic bacteria. Appl Soil Ecol 133:12–23

Evtushenko LI, Dorofeeva LV, Subbotin SA, Cole JR, Tiedje JM (2000) *Leifsonia poae*. gen. nov., sp. nov., isolated from nematode galls on *Poa annua*, and reclassification of "*Corynebacteriujm aquaticum*" Leifson 1962 as *Leifsonia aquatica* (ex Leifson 1962) gen. nov., nom. rev. comb. nov. and *Clavibacter xyli*, Davis et al., 1984, with two subspecies as *Leifsonia xyli* (Davis et al., 1984) gen. nov. comb. nov. Int J Syst Evol Micr 50:371-380

Faria RS, Cia MC, Monteiro-Vitorello CB, Azevedo RA, Camargo LEA (2020) Characterization of genes responsive to osmotic and oxidative stresses of the sugarcane bacterial pathogen *Leifsonia xyli* subsp. *xyli*. Braz J Microbiol 51(1):77–86

Fernandes Júnior AR, Ganem Júnior EDJ, Marchetti LBL, Urashima AS (2010) Avaliação de diferentes tratamentos térmicos no controle do raquitismo-da-soqueira em cana-de-açúcar. Trop Plant Pathol 35(1):060–064

Fu YH, Wei JJ, Pan YB, Zhou X, He EQH, Liu R, Huang H, Lu JJ, Liu FZ (2019) Comparative analysis reveals changes in transcriptomes of sugarcane upon infection by *Leifsonia xyli* subsp. *xyli*. J Phytopathol 167(11–12):633–644

Gagliardi PR, Camargo LEA (2009) Resistência de variedades comerciais de cana-de-açúcar ao agente causal do raquitismo-da-soqueira. Cienc Rural 39(4):1211–1214

Gillaspie AG, Davis MT, Harris RW, Lawson RH (1981) Isolation and pathogenicity of the ratoon stunting disease bacterium. Int Sugar J 83:324–326

Gillaspie AGJ, Teakle DS (1989) Ratoon stunting disease. In: Ricaud C, Egan AG, Gillaspie AGJ, Hughes CG, eds. Diseases of Sugarcane. Major Diseases. Amsterdam, Netherlands: Elsevier, 59–80

Gillis M, Kersters K, Hoste B, Janssens D, Kroppenstedt RM, Stephom MP, Teixeira KRS, Döbereiner J, Ley J (1989) *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugar cane. Int J Syst Bacteriol 39:361–364

Harrison J, Davis MJ (1988) Colonization of vascular tissues by *Clavibacter xyli* subsp. *xyli* in talks of sugarcane cultivars differing

in susceptibility to ratoon stunting disease. Phytopathology 78:722–727

Hendre RR, Iyor RS, Kotwalm M, Kluspe SS, Mascarenhas AF (1983) Rapid multiplication of sugarcane by tissue culture. Sugarcane 1:5–8

Hughes CG, Steindl DRL (1956) Some further developments in the study of ratoon stunting disease in Queensland. Proceedings of the International Society of Sugar Cane Technologists 9:1012–1022

Maron PA, Mougel C, Ranjard L (2011) Soil microbial diversity: methodological strategy, spatial overview and functional interest. C R Biol 334(5–6):403–411

Matteoli FP, Olivares FL, Venancio TM, Rocha LO, Irineu LESS, Canellas LP (2020) Chapter 23 – *Herbaspirillum*. In: Amaresan N, Kumar MS, Annapurna K, Kumar K, Sankaranarayanan A (eds), Beneficial Microbes in Agro-Ecology. Academic Press 493–508

Monteiro RA, Balsanelli E, Wassem R et al (2012) *Herbaspirillum*-plant interactions: microscopical, histological and molecular aspects. Plant Soil 356(1–2):175–196

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15(3):473–497

Muthukumarasamy R, Govindarajan M, Vadivelu M, Revathi G (2006) N-fertilizer saving by the inoculation of *Gluconacetobacter diazotrophicus* and *Herbaspirillum* sp. in micropropagated sugarcane plants. Microbiol Res 161(3):238–245

Olivares FL, James EK, Baldani JI, Döbereiner J (1997) Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. New Phytol 135(4):723–737

Oliveira ALM, Stoffels M, Schmid M, Reis VM, Baldani JI, Hartmann A (2009) Colonization of sugarcane plantlets by mixed inoculations with diazotrophic bacteria. Eur J Soil Biol 45(1):106–113

Oliveira MM, Ramos ETA, Drechsel MM, Vidal MS, Schwab S, Baldani JI (2018) Gluconacin from *Gluconacetobacter diazotrophicus* PAL5 is an active bacteriocin against phytopathogenic and beneficial sugarcane bacteria. J Appl Microbiol 125(6):1812–1826

Pellegrini M, Ercole C, Di Zio C, Matteucci F, Pace L, Del Gallo M (2020) *In vitro* and *in* planta antagonistic effects of plant growth-promoting rhizobacteria consortium against soilborne plant pathogens of *Solanum tuberosum* and *Solanum lycopersicum*. FEMS Microbiol Lett 367(13):fnaa099

Ponte ECD, Silveira SFD, Carneiro Jr. JDB, Lima RMPD (2010) Incidence of *Leifsonia xyli* subsp. *xyli* in sugarcane multiplication areas of Espírito Santo, South of Bahia, and Western of Minas Gerais States. Summa Phytopathol 36(4):313–321

Quecine MC, Silva TM, Carvalho G, Saito S, Mondin M, Teixeira-Silva NS, Camargo LEA, Monteiro-Vitorello CB (2016) A stable *Leifsonia xyli* subsp. *xyli* GFP-tagged strain reveals a new colonization niche in sugarcane tissues. Plant Pathol 65(1):154–162

Reis FB Jr, Silva LG, Reis VM, Döbereiner J (2000) Ocorrência de bactérias diazotróficas em diferentes genótipos de cana-de-açúcar. Pesqui Agropecu Bras 35:985–994

Reis VM, De Oliveira ALM, Da Silva MF, Olivares FL, Baldani JI, Boddey RM, Urquiaga S (2008) Inoculants for sugar cane: the scientific bases for the adoption of the technology for biofuel production. In Biological nitrogen fixation: towards poverty alleviation through sustainable agriculture 67–68

Reis VM, Olivares FL, Oliveira ALM, Reis FB Jr, Baldani JI, Döbereiner J (1999) Technical approaches to inoculate micropropagated sugarcane plants with *Acetobacter diazotrophicus*. Plant Soil 206:205–211

Ricaud C (1968) Ratoon stunting disease. Mauritius Sugar Industry Research Institute. Port Louis. Annual Report 54

Schmidt MA, Balsanelli E, Faoro H, Cruz LM, Wassem R, de Baura VA, Fungaro MH (2012) The type III secretion system is necessary for the development of a pathogenic and endophytic interaction between *Herbaspirillum rubrisubalbicans* and Poaceae. BMC Microbiol 12(1):98

Serrato RV, Sassaki GL, Cruz LM, Carlson RW, Muszyński A, Monteiro RA, Iacomini M (2010) Chemical composition of lipopolysaccharides isolated from various endophytic nitrogen-fixing bacteria of the genus *Herbaspirillum*. Can J Microbiol 56(4):342–347

Sreenivasan J, Sreenivasan TV (1984) In vitro propagation of a *Saccharum officinarum* (L.) and *Sclerostachya fusca* (Roxb.) A. Camus hybrid. Theor Appl Genet 67(2):171–174

Sun SR, Chen JL, Duan YY, Chu N, Huang MT, Fu HY, Gao SJ (2019) Improved primers for the specific detection of *Leifsonia xyli* subsp. *xyli* in sugarcane using a conventional PCR assay. Plant Dis 103(12):3251–3258

Urashima AS, Ganem EDJ Jr, Marchetti LBL, Gagliardi PR (2010) Incidence of *Leifsonia xyli* subsp *xyli* in sugarcane crops from São Paulo state. Summa Phytopathol 36(4):322–328

Urashima AS, Grachet NG (2012) Métodos de detecção de *Leifsonia xyli* subsp. *xyli* e efeito da termoterapia na brotação das gemas de diferentes variedades de cana-de-açúcar. Trop Plant Pathol 37(1):57–64

Urashima AS, Silva MF, Coraini NF, Gazaffi R (2020) Temporal incidence of *Leifsonia xyli* subsp. *xyli* in sugarcane propagating materials of Brazilian cultivars. Crop Protection 128:104976

Urashima AS, Silva MF, Correa JJ, Moraes MC, Singh AV, Smith EC, Sainz MB (2017) Prevalence and severity of ratoon stunt in commercial Brazilian sugarcane fields. Plant Dis 101(5):815–821

Van Elsas JD, Chiurazzi M, Mallon CA, Elhottovā D, Krištůfek V, Salles JF (2012) Microbial diversity determines the invasion of soil by a bacterial pathogen. Proc Natl Acad Sci 109(4):1159–1164

Wu Q, Pan YB, Zhou D, Grisham MP, Gao S, Su Y, Guo J, Xu L, Que Y (2018) A comparative study of three detection techniques for *Leifsonia xyli* subsp. *xyli*, the causal pathogen of sugarcane ratoon stunting disease. Biomed Res Int 2018:1–11

Young AJ (2016) Possible origin of ratoon stunting disease following interspecific hybridization of *Saccharum* species. Plant Pathol 65(9):1403–1410

Zhang X, Chen M, Liang Y, Xing Y, Yang L, Chen M, Comstock JC, Li Y, Yang L (2016) Morphological and physiological responses of sugarcane to *Leifsonia xyli* subsp. *xyli* infection. Plant Dis 100(12):2499–2506

Zhu K, Yuan D, Zhang XQ, Yang LT, Li YR (2018) The physiological characteristics and associated gene expression of sugar cane inoculated with *Leifsonia xyli* subsp. *xyli*. J Phytopathol 166(1):44–52