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ORIGINAL PAPER

Exploitation of new endophytic bacteria and their ability to promote sugarcane growth and nitrogen nutrition

Adriana Parada Dias da Silveira · Raquel de Paula Freitas Iório · Fernanda Castro Correia Marcos · Ana Olívia Fernandes · Silvana Aparecida Creste Dias de Souza · Eiko Eurya Kuramae · Matheus Aparecido Pereira Cipriano

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Abstract Few studies have evaluated endophytic bacteria in relation to plant growth promotion, nitrogen uptake and biological control. The aim of this study was to molecularly and physiologically characterize thirteen endophytic bacteria strains, evaluate their biological control properties and their ability to promote plant growth and plant N nutrition. All the strains produced indole acetic acid and promoted increase of plant biomass, N accumulative amount and N-use efficiency index. None of the strains carries the *nif*H gene. Four strains stimulated plant nitrate reductase activity, four solubilized phosphate, nine produced siderophores and none produced HCN. Seven strains inhibited *Bipolaris sacchari* growth and one was antagonistic to *Ceratocystis paradoxa*. The pathogens were inhibited by the production of diffusible and volatile metabolites by the bacterial strains. Moreover, this is the first study to demonstrate the effect of *Delftia acidovorans* on sugarcane plant growth, nitrogen metabolism improvement and antagonism to *B. sacchari*. The most efficient strains in promoting plant growth and exhibiting antagonistic activities towards fungal pathogens were *Herbaspirillum frinsingense* (IAC-BECa-152) and three *Pantoea dispersa* strains (IAC-BECa-128, IAC-BECa-129, and IAC-BECa-132). These bacteria show potential to be used as inoculants for sustainable agricultural management, mainly at the seedling production phase.

Keywords *Delftia acidovorans* · Fungal pathogen antagonism · Indole acetic acid · Nitrate reductase · Plant growth-promoting bacteria · *Saccharum* sp.

A. P. D. da Silveira (✉) · R. P. F. Iório · F. C. C. Marcos · A. O. Fernandes · M. A. P. Cipriano
Center of Soil and Environmental Resources, Agronomic Institute/IAC, P.O. Box-28, Campinas, SP 13010-970, Brazil
e-mail: apdsil@iac.sp.gov.br

S. A. C. D. de Souza
Center of Sugarcane, Agronomic Institute/IAC, Ribeirão Prêto, SP 14001-970, Brazil

E. E. Kuramae
Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands

Introduction

Beneficial microorganisms that inhabit the soil and influence plant development are known as plant growth-promoting bacteria and their effects have been studied worldwide (Finkel et al. 2018). The endophytic bacteria that colonize and live in internal plant tissue have also been extensively explored in different plants (Hardoim et al. 2015; Kumar et al. 2017). These microorganisms promote plant growth through different mechanisms, such as biological nitrogen fixation (BNF), increase of nitrate reductase activity,

production and release of plant hormones (auxins; indole acetic acid, gibberellins and cytokinins), phosphate solubilization, suppression of pathogens through siderophore production, synthesis of antibiotic, induction of systemic resistance, production of diffusible and volatile organic compounds, such as hydrocyanic acid (Olanrewaju et al. 2017) and others. Endophytic bacteria are also able to produce enzymes, such as ACC (1-aminocyclopropane-1-carboxylate) deaminase, that can promote plant growth by lowering plant ethylene levels (Rashid et al. 2012; Glick 2014). Thus, the use of plant growth-promoting endophytes is of great interest to enhance plant growth and protect plant from pathogens (Kruasuawan and Tramchaipen 2016).

In the last few decades, studies with diazotrophic bacteria, from the classical diazotroph *Acetobacter*, obtained from sugarcane (Cavalcante and Döbereiner 1988), to the endophytic *Achromobacter insolitus*, able to improve growth and N metabolism of wheat plants (Silveira et al. 2016), have intensified due to their potential as agents of growth promotion and plant protection (Marques et al. 2008). Several studies demonstrated the positive effect of endophytic bacteria on plants of economic interest, such as banana (Patel et al. 2017), maize (Alves et al. 2015), tomato (Upreti and Thomas 2015), groundnut (Dhole et al. 2016) and others. In sugarcane crop, the *Acetobacter* (Dong et al. 1995), *Pantoea* (Quecine et al. 2012) and *Gluconacetobacter* (James et al. 2001) bacteria promote beneficial effects on plant growth due to BNF, indole acetic acid (IAA) production and others mechanisms. Studies have shown that plant genetic factors contribute to increase plant growth efficiency with such microorganisms and that establishment of an endophyte in the plant can cause plant physiological changes that modulate plant growth and development (Conrath et al. 2006; Carvalho et al. 2006).

Brazil is the world's largest sugarcane producer, and the management of this crop has changed due to the expansion of sugarcane culture in the country. One of the management strategies is the use of commercial inoculants, composed of bacteria strains, such as endophytic bacteria that can stimulate the plant growth (Oliveira et al. 2006; Pandey et al. 2017). Seedlings production, one of main stages of cropping system, is conducted in nurseries via micropropagation and mini-stalks using organic substrates, which can favour the beneficial bacteria inoculation. It is known that endophytic microorganisms community that inhabit sugarcane tissues is more diverse than previously thought (Souza et al. 2016). It can make bacterial strains introduction more difficult due to higher space and niches competition. It may justify the importance to search for different and more efficient bacterial plant-promoting strains.

Endophytic bacterial strains can benefit sugarcane from the seedlings stage until field cropping. Sugarcane production can dramatically be reduced by pathogens, such as *Ceratocystis paradoxa* and *Bipolaris sacchari* (Girard and Rott 2000; Bournival et al. 1994). Finding endophytes with antagonist properties against these pathogens would be of great interest (Fávaro et al. 2012). In this aspect, strains with multiple characteristics in regard to plant growth promotion and biological control properties are still limited.

Despite the finding that there is a great diversity of bacteria that endophytically inhabit sugarcane tissues (Souza et al. 2016), there are few studies that have evaluated endophytic bacteria in relation to plant growth promotion, nitrogen uptake and biological control. In addition, the search for new bacterial strains that meet the inoculants market is constant, since it is known that strains can lose their efficacy (Meena et al. 2017), requiring their substitution. The inoculant market is growing worldwide, especially in Brazil, and bacteria, mainly endophytic with multiple beneficial characteristics, such as growth promotion and antagonism to pathogens, should be prioritized for use in inoculant formulations.

In this study sugarcane endophytes were evaluated for their ability to promote plant growth and improve plant N nutrition. Bacterial endophytes were characterized molecularly (16S rRNA gene sequencing and the presence of *nif*H) and physiologically (inorganic phosphate solubilization; siderophore, IAA, antifungal hydrocyanic acid and, diffusible and volatile organic compounds production). We hypothesized that endophytic bacteria trigger beneficial changes on sugarcane nitrogen metabolism which turns in plant growth promotion.

Materials and methods

All strains were obtained from the Agronomic Institute (Instituto Agronômico—IAC—Campinas, São Paulo, Brazil) Culture Collection, isolated from

healthy stems and roots of sugarcane cultivated under field cropping. Based on a previous screening for sugarcane seedling growth-promotion (Freitas 2011), thirteen endophytic bacteria strains were selected from 162 strains.

This study was divided into two parts. In part one, we performed a greenhouse experiment to evaluate the effects of endophytic bacteria on sugarcane seedlings in regard to plant nitrogen concentration, accumulation and use-efficiency index, and plant nitrate reductase activity. In part two, we characterized the strains by *nif*H gene presence, sequencing the 16S rRNA gene, and evaluated the strains for beneficial bacterial traits.

Part 1: greenhouse experiment and nitrogen analysis

The strains were evaluated on micropropagated sugarcane plants in a greenhouse experiment. The plantlets were obtained from the apical meristem of the variety IACSP95-5000 and cultivated in vitro (Sreenivasan and Sreenivasan 1984) for 70 days and then transferred to 50 mL flasks containing 15 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Nitrogen and other salts were used at a tenth of the recommended concentration, and no growth hormones were added to the MS medium. The clumps of micropropagated sugarcane were divided into four and transferred to flasks with MS medium using the same composition.

We prepared each bacterial inoculum by cultivating the strains on dextrose, yeast, glutamate (DYGS—Döbereiner et al. 1995) medium for 7 days at 30 °C. The inoculum density was adjusted to 10^8 colony-forming units (CFU) mL^{-1}. The plantlets were inoculated with 0.1 mL of bacterial suspension and placed in a growth chamber at 75% humidity, 14 h light of 60 m^2 lx intensity and 10 h of darkness at 27 °C for 7 days. After this period, the plantlets were transferred to pots containing 500 mL of sterilized commercial substrate (Carolina Soil®—composed by sphagnum peat, expanded vermiculite, dolomitic limestone and agricultural gypsum) and held in a greenhouse. The plantlets were covered with a plastic screen to reduce the light incidence by 60%. During the first month, only sterile water was used for irrigation; thereafter, a low-N nutrient solution (2 mL of solution A and 3 mL of solution B per liter; solution A: 200 g calcium nitrate, 250 g calcium chloride and 20 g ConMicrosStandart®perliter; solution B: 200 g potassium nitrate, 150 g monopotassium phosphate, 300 g magnesium sulphate and 100 g potassium chloride per liter) was added to the pots. After 2 months, a new division of clumps was performed, and the plants were transferred to 1 L pots containing sterilized commercial substrate (Carolina Soil®) and reinoculated (2 mL) with the strains. During the first month, only sterile water was used for irrigation, and during the subsequent months, a low-N nutrient solution was used. After 2 months, the plastic screen was removed. After 4 months, leaf samples were collected to determine nitrate reductase activity and, subsequently, the plants were harvested. The other plants were dried at 60 °C to determine root and shoot dry matter (five replicates per treatment). The shoot dry matter was ground and homogenized to determine the nitrogen concentration by micro-Kjeldahl method (Bremner 1965), the cumulative amount of N and the efficiency index of N utilization (Siddiqi and Glass 1981).

The nitrate reductase activity was determined by placing 200 mg of plant tissue discs in 5 mL of buffered substrate (200 mM KNO_3 in 50 mM phosphate buffer, pH 7.5) containing 0.5% Tween-20, according to Reed et al. (1980). The activity was measured by the absorbance at 540 nm.

Part 2: strains characterization

Phosphate solubilization ability

The phosphate solubilization ability of the strains was first analyzed by a qualitative test using a procedure that evaluates the solubilization of inorganic phosphate ($CaHPO_4$) on solid medium (Katznelson and Bose 1959). The strains that developed a clear halo surrounding the colonies were considered to be phosphate solubilizers. The level of solubilization was quantified in liquid medium containing 5 g $CaHPO_4$ L^{-1} as the only source of phosphorus (Murphy and Riley 1962; Nautiyal 1999). The amount of dissolved phosphorus was measured by atomic emission plasma spectrometry. The level of solubilization was also related to the total protein content.

Total protein determination

Total protein of the strain suspensions was determined using the method of Lowry et al. (1951) and modified by Rodrigues (2004). To 0.1 mL of the sample, 0.4 mL of sterile distilled H_2O and 0.5 mL of 1 M NaOH were added. The suspension was shaken and placed in a water bath for 5 min at 100 °C to lyse the cells; then, after the samples had reached room temperature, 0.25 mL of Lowry reagent was added to the sample, which was incubated for 10 min in the dark. Subsequently, 0.5 mL of Folin–Ciocalteau reagent (2:1 dilution of the reagent in distilled H_2O) was added, and the sample was shaken; then, the suspension was again incubated in the dark for 30 min. After this, the absorbance was measured at 750 nm. Protein concentration was calculated based on a standard curve prepared using Bovine Serum Albumin (BSA).

Indole acetic acid (IAA) quantification

IAA production was quantified according to Kojima (1996) adapted for microbial cultures. The strains were grown in 250 mL of DYGS medium containing 100 mg L-tryptophan L^{-1} and incubated for 72 h under constant stirring in the dark at 30 °C. The sample was centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatant was filtered through a 0.22 µm Millipore filter, and a 5 mL aliquot was acidified using 1 N HCL to pH 2.5 and partitioned three times with diethyl ether using a separating funnel. The extract was evaporated and suspended in 300 µL of methanol. The samples were analyzed by high-performance liquid chromatography (HPLC) using a C18 reversed-phase column. The results were expressed in µg IAA per mg total protein.

Siderophore production

Siderophore production was measured using the method of Schwyn and Neilands (1987), in which the dye is released from a dye-iron complex when a ligand sequesters the iron complex; this reaction causes a color change from blue to yellow-orange. The dye used was chromeazurol S (CAS), and the binder was one or more of the siderophores found in the culture supernatants of the bacterial strains.

Hydrocyanic acid production (HCN)

The capacity of HCN production by the strains was evaluated according to Bakker and Schippers (1987). The strains were grown on PDA medium supplemented with 4.4 g L^{-1} of glycine to stimulate the production of hydrocyanic acid. A filter paper impregnated with 0.5% picric acid and 2% Na_2CO_3 was laid on a cover plate, which was then sealed with Parafilm and incubated at 28 °C. Color change of the paper from yellow to orange indicated the production of hydrocyanic acid by the strain.

Antifungal activity assay by paired culture (PC), volatile organic compound (VOC) and diffusible compound (DC) tests

The antagonistic properties of the strains were evaluated for two pathogenic fungi, *B. sacchari* and *C. paradoxa*, using the paired culture (PC) method. The fungi were cultured in Petri dishes containing potato dextrose agar (PDA) medium for 7 days (*C. paradoxa*) and 10 days (*B. sacchari*). For the PC tests, the bacterial strains (grown in DYGS liquid medium for 4 days) were arranged as streaks on one side of the Petri dish with PDA medium; a 5 mm PDA culture medium containing the pathogen mycelial disc was placed on the opposite side. The control treatment contained only the mycelial disc of the pathogen. Antagonistic action was determined by the formation of a zone of inhibition (halo without mycelial growth). Strains that inhibited pathogen growth were tested for the production of volatile organic compounds (VOCs) and diffusible compounds (DC). To analyze the VOCs, the technique of overlapping plates proposed by Dick and Hutchinson (1966) was used. A disc of pathogen culture was placed at the center of a Petri dish containing PDA, and 0.1 mL of the antagonist was inoculated on another plate. The Petri dishes containing the pathogen was superimposed over the plate containing the antagonist, and both were wrapped in plastic film and incubated at 28 °C. The production of DC was analyzed using the cellophane method (Gibbs 1967); the antagonist culture was grown in DYGS liquid medium, and the Petri dish containing PDA medium was covered with sterile cellophane overlaid with a sterile filter paper disc. In the center of each plate, 0.1 mL of the antagonist was inoculated on the surface of the filter paper. After

5 days, the filter paper and the cellophane with the colony of antagonists were removed, and a disc of PDA containing the pathogen was transferred to the center of the plate. Both tests were evaluated after total medium coverage by the mycelium of the pathogen in the control treatment by measuring the pathogen mycelial growth. The tests were performed using five replicates per treatment, which included a control treatment containing only the pathogen.

Amplification of the nifH gene

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The presence of the *nif*H gene was determined using the polymerase chain reaction (PCR) and the *nif*H forward and reverse primers Polf (TGC-GAY-CCS-AAR-GCB-GAC-TC) and Polr (ATS-GCC-ATC-ATY-TCP-CCG-GA), and PPf (GCA-AGT-CCA-CCA-CCT-CC) and PPr (TCG-CGT-GGA-CCT-TGT-TG), as described by Ueda et al. (1995). The PCR method was as follow: denaturation at 94 °C for 4 min., followed by 35 cycles (94 °C for 1 min, 50 °C for 45 s and 72 °C for 2 min), with a final extension at 72 °C for 4 min. The amplification products were electrophoresed on a 1.5% agarose gel in 1xTAE buffer.

16S rRNA gene amplification and sequencing

Bacterial genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. We amplified the 16S rRNA gene from all the strains using the primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) with the PCR conditions as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, with a final extension at 72 °C for 10 min. The amplification products were electrophoresed on a 1.0% agarose gel in 1xTE buffer, purified using the QIAquick Gel Extraction Kit (QIAGEN), cloned into the pGEM T-plasmid (pGEM-T Easy Kit, Promega) and transformed into JM109 *Escherichia coli* competent cells according to the manufacturer's instructions. For each amplified and transformed fragment, eight single colonies were sequenced using the BigDye Terminator v3.1 Kit and an automated DNA capillary sequencer (ABI PRISM 3700 DNA Analyser, PE Applied Biosystems, HITACHI); the primers used were T7 (TAATACGACTCACTATAGGG) and SP6 (ATTTAGGTGACACTATAGAA). For each fragment, all 16S rRNA gene partial sequences obtained were assembled into a contig using the Phred/Phrap/CONSED program (repeat stringency 0.5) (Ewing et al.1998; Gordon et al. 1998). The sequences were identified by comparing the contiguous 16S rRNA gene sequences obtained with 16S rRNA sequence data from the references and type strains available in the GenBank and RDP (Ribosomal Database Project, Wisconsin, USA) data bases using BLASTn and the RDP classifier, respectively. The phylogenetic tree was obtained by aligning (ClustalW) nearly full-length 16S rRNA gene sequences and reference sequences and using the neighbour-joining method (Saitou and Nei 1987); bootstrapping values were based on 1000 repetitions.

Statistical analysis

The results were subjected to analysis of variance, and the means were tested using the Scott–Knott test at 5% ($P < 0.05$).

Results

Part 1: greenhouse experiment with sugarcane plantlets

All the strains contributed to increase dry matter compared with the non-inoculated control treatment. Except for IAC-BECa-105, all strains improved shoot dry mass, and seven of them improved root dry mass (Table 1). Four strains (IAC-BECa-023, IAC-BECa-126, IAC-BECa-129, IAC-BECa-152) promoted an increase in shoot N increase in shoot N concentration and twelve strains increased shoot N cumulative amount. Plants inoculated with all strains, excepted strain IAC-BECa-023, showed higher values of efficiency index of N utilization than the control plant. The activity of nitrate reductase in leaves was significantly higher in plants inoculated with four different strains (IAC-BECa-026, IAC-BECa-152, IAC-BECa-101 and IAC-BECa-105) than in the control plants (Table 1).

Table 1 Shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM), nitrogen content (NC), N cumulative amount (NCA), N use efficiency index (NUE) and nitrate reductase activity (NR) of sugarcane non-inoculated (control) or inoculated with different endophytic bacterial strains

Treatments	SDM (g)	RDM (g)	TDM (G)	NC (g Kg^{-1})	NCA (mg $plant^{-1}$)	NUE (g^2g^{-1})	NR (nmol g^{-1})
Control	8.09	1.81*	9.90	12.32	99.31	0.66	49.2
IAC-BECa-046	11.46*	2.14*	13.60*	11.33	130.52*	1.01*	50.0
IAC-BECa-023	10.63*	2.60*	13.23*	13.41*	143.46*	0.79	36.7
IAC-BECa-026	11.02*	2.14	13.16*	12.19	134.14*	0.90*	113.3*
IAC-BECa-126	12.42*	2.08	14.50*	12.63*	156.67*	0.99*	26.7
IAC-BECa-128	11.78*	2.27*	14.05*	12.05	141.91*	0.98*	40.0
IAC-BECa-129	13.23*	2.13	15.36*	12.56*	166.59*	1.05*	46.7
IAC-BECa-132	11.25*	1.94	13.19*	12.08	136.95*	0.93*	40.0
IAC-BECa-152	12.09*	2.25*	14.34*	12.53*	151.69*	0.96*	116.7*
IAC-BECa-162	11.80*	1.97b	13.77*	11.68	137.24*	1.01*	43.3
IAC-BECa-098	11.57*	2.79*	14.37*	11.20	129.27*	1.04*	30.0
IAC-BECa-101	13.44*	2.79*	16.23*	10.89	146.40*	1.24*	93.3*
IAC-BECa-102	11.77*	2.50*	14.27*	11.81	136.57*	1.02*	63.3
IAC-BECa-105	10.41	2.42*	12.83*	11.47	119.25	0.91*	100.0*

*Values statistically different from the control treatment by Scott Knot test ($P < 0.05$)

Part 2: strains characterization

All 13 strains produced IAA at levels between 0.04 and 5.37 µg of IAA per mg of protein (Table 2). Four strains (IAC-BECa-128, 129, 132 and 162) exhibited the ability to solubilize phosphate by qualitative and quantitative tests, and nine strains (IAC-BECa-023, 026, 126, 128, 129, 132, 152, 102 and 105) produced siderophores. None of the strains produced HCN. The *nif*H sequence did not be amplified using the Pol primers in any of the thirteen strains, but positive detection of *nif*H was verified for the positive controls (data not shown).

Seven strains (IAC-BECa-023, 128, 129, 132, 098, 101, 102) that showed an inhibition zone of *B. sacchari* mycelium growth in the test of the PC also acted as antagonists for DC and VOC tests (Table 3). For the pathogen *C. paradoxa*, only the strain IAC-BECa-129 inhibited mycelial growth for all antifungal tests.

The 16S rRNA gene sequences of 13 strains were compared with the 16S rRNA of phylogenetically closely related bacteria (Fig. 1). The strains were clustered into seven different groups. IAC-BECa-105 is in the cluster of *Methylobacterium* species and IAC-BECa-023 is in the *Delftia* species cluster. IAC-BECa-152 is similar to *Herbaspirillum frisingense*, while IAC-BECa-046 is similar to *Burkholderia caribensis*. Strain IAC-BECa-162 clusters with different species of *Burkholderia*; IAC-BECa-098 was placed in the *Pseudomonas* species cluster; and IAC-BECa-026, IC-BECa-128, IAC-BECa-132 and IAC-BECa-129 were clustered with *Pantoea*. The strains IAC-BECa-102, IAC-BECa-101 and IAC-BEC-126 were in the *Enterobacter* cluster. The final sequences of the studied strains are available in GenBank (under the accession numbers in Table 2).

Discussion

Some of the sugarcane endophytic strains characterized in this study have already been described as plant growth-promoting bacteria. The strains IAC-BECa-152, similar to *H. frisingense,* and IAC-BECa-046, to *B. caribensis,* which have beneficial characteristics related plant growth (Kirhhof et al. 2001; Marcos et al. 2016, Schlemper et al. 2018), improved, respectively, 45 and 37% of the total dry mass (TDM) according the relative efficiency (Table 4). The other strains were clustered in different groups of *Methylobacterium* and *Enterobacter cloacae.* The strains were also clustered

Table 2 Indole acetic acid production (IAA), phosphate solubilization (PS), siderophore and hydrocyanic acid (HCN) production, and amplification of *nif*H. The accession number of the 16S rRNA gene sequences deposited in NCBI database. Values with the same lower case do not differ by the Scott-Knott test ($P < 0.05$)

Strains	Closest match according to the 16S rRNA gene sequence	IAA	PS	Siderophore	HCN	*Nif*H	Accession number
IAC-BECa-046	*Burkholderia caribensis*	2.47c	nd	−	–	–	JX155399
IAC-BECa-023	*Delftia acidovorans*	0.49de	nd	+	–	–	JX155411
IAC-BECa-026	*Pantoea dispersa*	0.04e	nd	+	–	–	JX155413
IAC-BECa-126	*Enterobacter asburiae*	0.34de	nd	+	–	–	JX155406
IAC-BECa-128	*Pantoea dispersa*	0.07e	1.02 c	+	–	–	JX155407
IAC-BECa-129	*Pantoea dispersa*	0.07e	1.04 c	+	–	–	JX155408
IAC-BECa-132	*Pantoea dispersa*	3.73b	2.08 b	+	–	–	JX155401
IAC-BECa-152	*Herbaspirillum frinsingense*	0.98d	nd	+	–	–	JX155400
IAC-BECa-162	*Burkholderia*sp.	0.10e	3.98 a	−	–	–	JX155405
IAC-BECa-098	*Pseudomonas* sp.	5.37a	nd	−	–	–	JX155415
IAC-BECa-101	*Enterobacter cloacae* subsp. *Cloacae*	1.91c	nd	−	–	–	JX155417
IAC-BECa-102	*Enterobacter* sp.	1.91c	nd	+	–	–	JX155418
IAC-BECa-105	*Methylobacterium oryzae*	3.43b	nd	+	–	–	JX155419

Bacterial strains: absence (−) or presence (+) of ability

Nd not determined (absence of clear halo in culture medium)

with genera already known as endophytes of sugarcane, such as *Burkholderia*, *Pseudomonas* and *Pantoea* (Mendes et al. 2007; Shahid et al. 2017). The strain IAC-BECa-023, identified as *Delftia acidovorans*, a species which includes some strains that already have been described as plant growth-promoting bacteria of soybean and pea plants (Adesemoye et al. 2017) and also been isolated from endophytic tissues of sugarcane (Mehnaz et al. 2010). However, this is the first study to show the growth promotion effect of *D. acidovorans* on sugarcane seedling, with an improvement of 34% of the TDM.

Recently, endophytes have been tested in sugarcane in a mixed inoculum, which increased the plant nitrate reductase activity (Marcos et al. 2016), an effect related to an increase in nitrate uptake, mainly under low N concentrations (Donato et al. 2004). The inoculation of IAC-BECa-152, 105, 026 101 strains in micropropagated sugarcane resulted in higher plant nitrate reductase activity up to 137, 103, 130 and 90% (Table 4).

BNF is attributed to endophytic bacteria as a benefit to host plant development; however, it is known that N fixation is not the only benefit triggered by these microorganisms (Vacheron et al. 2013). In the present study, the selected strains, despite exhibiting potential as plant-growth promoters, did not fix N based on the absence of *nif*H gene amplification. Sevilla et al. (2001), while studying a *nif*H mutant of *G. diazotrophicus* PAL5 in sugarcane, verified that the plant growth stimulus might be associated with auxin production. The production of auxin is related to plant growth stimulation and the proliferation of secondary roots (Patten and Glick 2002). Microbe-plant associations may have a large influence on this production, especially during the early stages of development and rooting (Kröber et al. 2014). Microorganisms produce growth-promoting substances, including indole compounds that possess rings, such as auxins. IAA is the most active auxin and has been widely studied due to its role in promoting both fast responses, such as increased cell elongation, and slow responses, such as cell division and differentiation (Bailly et al. 2014; Cherif-Silini et al. 2016). The strains studied here are IAA producers, which may have contributed to plant growth. The ability to synthesize phytohormones, including IAA, is widely distributed amongst plant-associated bacteria (Duca et al. 2014), showing that the bacteria can trigger this effect in different plants such as leguminous vegetables (Ahmed et al. 2014), rice (Pittol et al. 2016), lettuce (Cipriano et al. 2016),

Table 3 Antagonism to fungal phytopathogens *Bipolaris sacchari* and *Ceratocystis paradoxa*—paired cultures (PC), diffusible compounds (DC) and VOCs

Strains	Antifungal activity against *Bipolaris sacchari*			Antifungal activity against *Ceratocystis paradoxa*		
	PC Growth inhibition	DC % inhibition	VOCs % inhibition	PC Growth inhibition	DC % inhibition	VOCs % inhibition
IAC-BECa-046	−	nd	nd	−	nd	nd
IAC-BECa-023	+	23.5	11.7	−	nd	nd
IAC-BECa-026	−	nd	nd	−	nd	nd
IAC-BECa-126	−	nd	nd	−	nd	nd
IAC-BECa-128	+	84.7	17.6	−	nd	nd
IAC-BECa-129	+	39.4	23.5	+	14.1	10.5
IAC-BECa-132	+	38.8	29.6	−	nd	nd
IAC-BECa-152	−	nd	nd	−	nd	nd
IAC-BECa-162	−	nd	nd	−	nd	nd
IAC-BECa-098	+	32.9	52.9	−	nd	nd
IAC-BECa-101	+	23.5	25.8	−	nd	nd
IAC-BECa-102	+	25.9	8.4	−	nd	nd
IAC-BECa-105	−	nd	nd	−	nd	nd

Absence (−) or presence (+) of antagonistic activity

Nd not determined

sugarcane (Rampazzo et al. 2018), chrysanthemum (Cipriano and Freitas 2018) and other crops.

In addition to the ability to produce phytohormones, nine strains produced siderophores, substances that may increase iron availability to the plant and inhibit the growth of pathogenic microorganisms (Compant et al. 2005). Four strains were able to solubilize phosphate, which is well described as plant growth-promoting trait in several host plants (Bonaldi et al. 2015; Kielak et al. 2016), including strains isolated from sugarcane (Xing et al. 2016), and thus, may have contributed to sugarcane-growth promotion.

Endophytic bacteria with antagonistic activities towards soil-borne fungal pathogens have already been reported (Vurukonda et al. 2018). Moreover, biological control by endophytes is evident in several crops, such as olives (Cabanás et al. 2014), tomato cultivars (Upreti and Thomas 2015) and rice (Verma et al. 2018). In sugarcane, *P. dispersa* is endophytically associated with this host plant and representatives of this genus can detoxify albicidins (a family of phytotoxins) produced by *Xanthomonas albilineans*, that causes sugarcane leaf scald disease (Zhang and Birch 1997; Ding and Melcher 2016). The plant growth-promoting bacteria of the genus *Delftia* also exhibited broad-spectrum antifungal activity to different pathogens such as *Fusarium*, *Colletotrichum*, *Rhizoctonia* and *Pythium* (Prasannakumar et al. 2015), but our data are the first to show activity against *B. saccharis* through volatiles compounds production. The inhibition of pathogens can occur through various mechanisms, including competition for nutrients, production of volatile organic compounds (VOCs) and non-volatile substances, competition for colonization sites and induction of systemic resistance (Eljounaidi et al. 2016; Khalaf and Raizada 2018). Selecting strains that inhibit the growth of more than one pathogen may provide support for the use of endophytes in disease suppression and plant-growth promotion. Several bacteria such as *Pseudomonas*, *Bacillus* and *Burkholderia* are antagonistic towards *Bipolaris* (Rafikova et al. 2016; Bach et al. 2016) and *Ceratocystis* species (Zhang et al. 2014). However, to date, there are no studies showing antagonism between endophytic bacteria and sugarcane pathogens such as *C. paradoxa* and *B. sacchari*. In the present study, we selected for the first time the endophytic bacterium *P. dispersa* (IAC-BECa-129) able to inhibit the mycelial growth of *B. sacchari* and *C. paradoxa* due to direct antagonism triggered by diffusible compound and

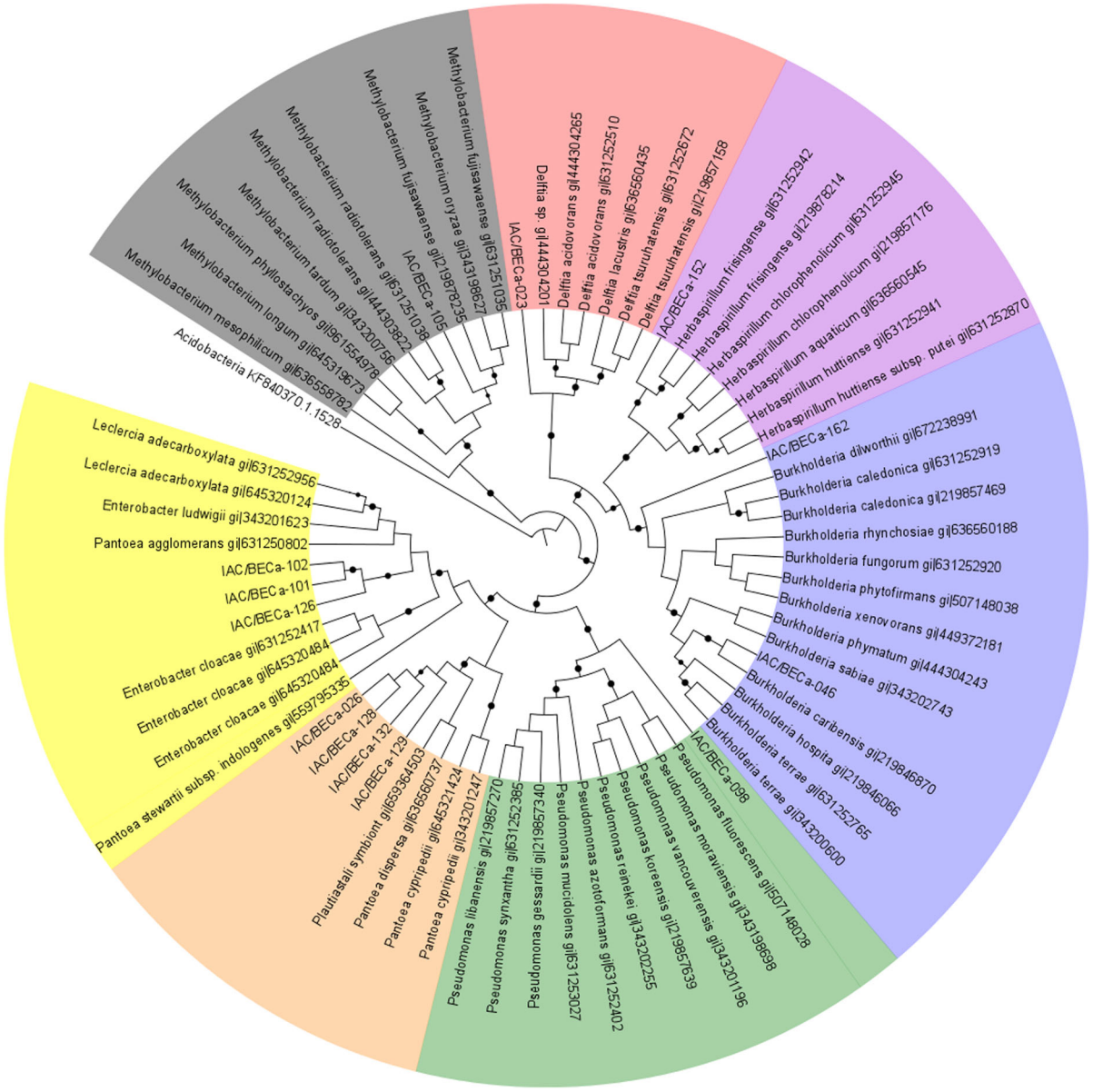

Fig. 1 Phylogenetic analysis of 13 sugarcane endophyte strains based on Neighbor joining method. Bootstrapping values were based on 1000 repetitions and are shown by black circles (> 75%). *Acidobacteria* is outgroup

VOC production (Table 3). Although the strains studied here do not produce HCN, a typical VOC, these strains were able to inhibit pathogen mycelial growth due to diffusible compounds and probably by producing other VOCs, not determined in this study, such as dimethyl sulphide and pyrazine (Hernández-León et al. 2015; Rybakova et al. 2016). As far as we know the strains *D. acidovorans* (IAC-BECa-023) and *P. dispersa* (IAC-BECa128, 129 and 132) are the first able to produce VOCs, besides diffusible compounds, revealing an opportunity to investigate the role of the selected strains in *B. sacchari* control.

All thirteen strains promoted up to 66% increases in shoot dry matter, 54% in root dry matter and 68% in N accumulation. This growth promotion, as well as the increase in N nutrition (as reflected by the N concentration, N cumulative amount, N utilization efficiency index and nitrate reductase activity), might be related

Table 4 Relative efficiency (%) of bacterial endophytes on sugarcane seedlings biomass production and N nutrition

Strains	Closest match according to the 16S rRNA gene sequence	SDM	RDM	TDM	NC	NCA	NUE	NR
IAC-BECa-046	*Burkholderia caribensis*	42	18	37	–	31	53	–
IAC-BECa-023	*Delftia acidovorans*	31	44	34	9	45	–	–
IAC-BECa-026	*Pantoea dispersa*	36	–	33	–	35	36	130
IAC-BECa-126	*Enterobacter asburiae*	54	–	47	3	58	50	–
IAC-BECa-128	*Pantoea dispersa*	46	25	42	–	43	49	–
IAC-BECa-129	*Pantoea dispersa*	64	–	55	2	68	59	–
IAC-BECa-132	*Pantoea dispersa*	39	–	33	–	38	41	–
IAC-BECa-152	*Herbaspirillum frinsingense*	49	24	45	2	53	46	137
IAC-BECa-162	*Burkholderia* sp.	46	–	39	–	38	53	–
IAC-BECa-098	*Pseudomonas* sp.	43	54	45	–	30	58	–
IAC-BECa-101	*Enterobacter cloacae* subsp. *cloacae*	66	54	64	–	47	88	90
IAC-BECa-102	*Enterobacter* sp.	46	38	44	–	38	55	–
IAC-BECa-105	*Methylobacterium oryzae*	29	34	30	–	20	38	103

Shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM), nitrogen content (NC), N cumulative amount (NCA), N use efficiency index (NUE) and nitrate reductase activity (NR)

to improved nutritional efficiency resulting from more efficient plant-bacteria interaction. Higher nutritional efficiency due to associations with microorganisms could be related to plant growth and improved nutrient absorption (Mantelin and Touraine 2004; Barretti et al. 2008). Indeed our results support that hypothesis since the plants with higher nutritional efficiency also obtained the higher values for biomass.

Endophytic bacteria that exhibit plant growth promotion and biological control features have the potential to be used as inoculants, potentially replacing — completely or partially — chemical fertilizers and pesticides. In addition, this technology is less expensive than chemical application and has a low environmental impact, allowing sustainable agricultural management. In this study, we add to knowledge about the interaction between endophytes and sugarcane, including antagonism tests related to important sugarcane diseases. The bacteria *H. frinsingense* (IAC-BECa-152), three *P. dispersa* strains (IAC-BECa-128, IAC-BECa-129, IAC-BECa-132) and *D. acidovorans* (IAC-BECa-023) exhibited the best results in regard to plant growth, production of compounds such as siderophore and IAA, ability to solubilize phosphate, VOC production and direct antagonism to *B. sacchari* and *C. paradoxa*.

Many studies, previously, reported the effects of endophytes on sugarcane and other crops regarding to strains characterization and antagonism to several pathogen. Although these studies have already shown the beneficial effect of *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Pantoea* and *Methylobaterium* in several cultures, this is the first study to show that the endophytic bacteria *Delftia dispersa* (strain IAC-BECa-023) is responsible for the growth of sugarcane seedlings and N metabolism improvement. Further studies with the present strains under field conditions will elucidate their impact on sugarcane and pathogen interactions. In summary, we broaden the knowledge of the growth-promoting bacteria, including the antagonistic potential triggered by volatile substances of endophytes strains to the pathogens *B. sacchari* and *C. paradoxa*.

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Authors contribution APDS conceived the study. APDS, RPFI, FCCM, AOF, SCS and MAPC performed the experiments. APDS, RPFI, EEK and MAPC wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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