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PLANT TISSUE CULTURE

Characterization of the endophytic bacteria from *in vitro* cultures of *Dendrocalamus asper* and *Bambusa oldhamii* and assessment of their potential effects in *in vitro* co-cultivated plants of *Guadua chacoensis* (Bambusoideae, Poaceae)

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Abstract
Bamboos (Bambusoideae, Poaceae) are multiple-purpose perennial grasses, which display a growing production chain in Brazil. One of the main bottlenecks is high-quality supplying of plantlets, then requiring efficient mass propagation methods, such as micropropagation. Contamination by microorganisms is recurrent in bamboo *in vitro* cultures, although some of those manifestations are considered endophytes harboring plant growth promotion potential. The isolation of endophytic bacteria from *in vitro* cultures of *Dendrocalamus asper* and *Bambusa oldhamii* was performed to assess their potential growth-promoting effect in co-cultivation with *in vitro* plants of *Guadua chacoensis*, an economically promising bamboo species. Among the total bacterial collection (32 isolates), all of them showed growth-promotion potential as indole compounds-producers. Sequences of 16S rRNA genes from eight selected isolates were newly generated, and the BLASTn similarity test recovered four bacterial genera (*Bacillus*, *Brevibacillus*, *Serratia*, and *Atlantibacter*) and six species. The co-cultivation experiment was carried out with three isolates selected based on their low- (Ba16), medium- (Ba03), and high-yield (Ba24) production of indole compounds, and Bayesian inferences strongly supported them as *Bacillus subtilis*, *Serratia marcescens,* and *Brevibacillus parabrevis*, respectively. The co-cultivation with three bacterial isolates, and their dilution levels, did not influence shoot or root growth and, however, did not cause apparent impairment for *G. chacoensis in vitro* cultures. Taken together, the isolation of endophytic microorganisms from field-growth bamboo clump and its co-cultivation with *in vitro* cultures of bamboos is possible, encouraging a continuous discovery and improvement of micropropagation techniques.

Keywords Bamboo micropropagation · Co-cultivation · Growth-promoting · Microbiome

Introduction

Bambusoideae subfamily (Poaceae) encompasses 127 genera and 1680 species classified in woody (Bambuseae and Arundinarieae tribes) and herbaceous lineages (Olyreae tribe), representing the main grass lineage with diversification in forest habitats (Soreng *et al.* 2017; Clark and Oliveira 2018). With a worldwide distribution, bamboos are multipurpose plants, providing crucial environmental, social, and economic benefits as important non-timber resources (Clark *et al.* 2015). Even though Brazil is one of the main centers of diversity and endemism in the Neotropics, bamboo exploitation is still not widespread, especially due to the abundant traditional timber industry and technological gaps related to the appropriate use of exotic and native species (Nogueira *et al.* 2017; Clark and Oliveira 2018).

One of the main limitations for the consolidation of the bamboo productive chain in Brazil is the difficulty of supplying plantlets on a large scale with high genetic and phytosanitary quality (Sánchez *et al.* 2011). Thus, new mass propagation methods are being developed to meet the increasing plantlet's demands, such as micropropagation (Mudoi *et al.* 2013; Singh *et al.* 2013; Sandhu *et al.* 2018), in which

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bamboo *in vitro* culture are frequently associated with the major problem of contamination by microorganisms (Leifert and Cassells 2001; Ramanayake *et al*. 2006).

Microorganisms' contamination in presumed axenic systems, especially by bacteria, is mainly reported as having harmful effects on *in vitro* plant development (Orlikowska *et al*. 2017). However, many of these microorganisms can have an endophytic nature capable of internally colonizing plant tissues without causing tissue or developmental damages to the host (Hardoim *et al*. 2008; Reinhold-Hurek and Hurek 2011). Within those microorganisms, some endophytic bacteria have remarkable potential benefits to the plant host as symbiotics relationships, either by direct or indirect pathways, such as biological nitrogen fixation, secretion of plant hormones, and mechanisms of disease resistance and adverse environmental tolerance (Mano and Morisaki 2008; Ramakrishna *et al*. 2019).

Due to the complex interaction of endophytes with their hosts, efforts are being taken place focusing on the understanding of functional biology and potential application of those microorganisms (Moshynets *et al*. 2012; Pérez-Montaño *et al*. 2014). Each plant species can host one or more endophytes, harboring diverse microbiological communities associated with different parts and stages of development of the plant, along with cultivation and ecological systems (Rosenblueth and Martínez-Romero 2006; Liu *et al*. 2017; Zheng and Lin 2020). Isolation of beneficial bacterial strains and *in vitro* plant co-culture, under controlled conditions, could promote symbiotic relationships towards plant growth and biotic and abiotic stress tolerance, process known as biotization or bio-priming (Nowak 1998; Lim *et al*. 2016; Mahmood *et al*. 2016).

A well-recognized endophytic bacterial stimulus is the auxin indole-3-acetic acid (IAA) biosynthesis, a phytohormone which plays crucial role in controlling many plant physiological processes (Duca *et al*. 2014; Moronta-Barrios *et al*. 2018; Zhang *et al*. 2019a). A wide range of plant-associated bacteria are reported as IAA producers, which could promote, in a dose-dependent manner, root growth and mitigate stress at *ex vitro* acclimatization phases of *in vitro* plants (Patten and Glick 2002; Kargapolova *et al*. 2020; Pace *et al*. 2020).

Although several endophytic bacterial communities have been identified in bamboo species (Han *et al*. 2009; Moshynets *et al*. 2012; Yuan *et al*. 2015; Liu *et al*. 2017; Zhang *et al*. 2019b; Singh *et al*. 2020; Zheng and Lin 2020), they are mostly treated as harmful contaminants in *in vitro* cultures (Nadha *et al*. 2012; Ray and Ali 2017; Ray *et al*. 2017; Leão *et al*. 2020), and little is known about their supposed role *in vitro* in plant growth. Thus, considering high contamination rates and the endophytic bacterial diversity in bamboo species, the identification of those microorganisms in presumed axenic *in vitro* cultures and assessment of their potential growth-promotion effect would be an important tool to overcome methodological limitations and to improve bamboo micropropagation methods (Ramanayake *et al*. 2006; Abreu-Tarazi *et al*. 2010; Singh *et al*. 2020).

Dendrocalamus asper and *Bambusa oldhamii* are bamboo species with remarkable commercial potential, and, in both genera, a great diversity of endophytes, including plant growth-promoting bacteria, was recently described (Benton 2015; Singh *et al*. 2020). In the present work, we characterized the bacterial isolates obtained from *in vitro* cultures of *B. oldhamii* and *D. asper*, using morphological culture parameters, production of indole compounds, and molecular techniques, and evaluated their growth-promoting effects *in vitro* co-culture of *Guadua chacoensis*, an economical promising bamboo species in southern Brazil.

Materials and Methods

Plant material and culture conditions The explants were collected from 7-old field-grow clumps in Santa Rosa de Lima, Santa Catarina, Brazil. *In vitro* cultures of *Dendrocalamus asper* (Schult. & Schult. f.) Backer ex K. Heyne and *Bambusa oldhamii* Munro were established using nodal segments as explants, which were subjected to disinfestation procedures prior to *in vitro* introduction, according to Santos *et al*. (2019). Since the employed disinfestation procedures were efficient for complete elimination of epiphytic bacteria, and then the cultures were considered aseptic, those that manifested late microorganism's growth were selected for isolation as putative endophytic bacterial strain.

Isolation and morphological characterization of bacterial isolates Endophytes bacterial colonies were isolated and purified by streaking-plating, on Luria Bertani (LB) solid media (Sigma-Aldrich, St. Louis, MO) for 24 to 48 h at 28°C, until morphological identification of a single colony was obtained. Morphological characteristics of isolated colonies, such as color, size, shape, elevation, border, transparency, structure, and Gram stain, were recorded in a matrix according to Rodina (1972).

Quantification of indole compounds Simultaneously to the morphological characterization, the isolates were subjected to qualitative and quantitative analysis of indole compounds (IC) production, following the spectrophotometric method based on Salkowski's reagent, with modifications (Glickmann and Dessaux 1995). The isolates were incubated, in triplicate, in 5 mL of LB liquid medium, at 30°C from 24 to 72 h in the Biochemical Oxygen Demand (BOD) chamber. Afterwards, the cultures were mixed with the Salkowski reagent (0.45% $FeCl_3$ (w/v) in 10.2 M H_2SO_4 (both from Sigma-Aldrich)), in a ratio of 1:1 (v/v), and kept for 30 min in dark at room temperature (25°C ± 2°C). The presence of IC

was visually evaluated by the development of pink color in the mixture the optical density was recorded at 540 nm in order to estimate IC concentration against the standard curve of synthetic IAA (in the range of 1 to 10 μg mL^{-1}), according to Radwan *et al.* (2005). Then, the bacterial isolates were classified into low-yield (IC ≤ 4 μg mL^{-1}), medium-yield (4 < IC ≤10 μg mL^{-1}), and high-yield categories (IC > 10 μg mL^{-1}) by comparing means of IC concentration.

Isolates' identification by 16s rRNA sequencing and phylogenetic inferences Among the total bacterial collection, eight isolates encompassing the three yield-based categories were selected for molecular characterization by 16S rRNA partial sequencing (Ba03, Ba05, Ba16, Ba18, Ba21, Ba24, Ba29, and Ba32). Bacterial genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen. Inc., Valencia, CA) and evaluated in 0.8% agarose gel electrophoresis (Sambrook and Russell 2001). Amplification of 16S rRNA gene was performed by polymerase chain reaction (PCR) using universal primers 27F/1492R (Lane 1991). Amplification products were examined by 1.5% agarose gel electrophoresis and further purified by differential precipitation with PEG8000 solution (20% PEG8000 and 2.5M NaCl (both from Sigma-Aldrich)) (Lis and Schleif 1975).

Sequencing reactions were carried out using the Big Dye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA), and further purified by the ethanol/EDTA/sodium acetate precipitation protocol. The bidirectional sequencing was performed at an ABI 3500xL automated sequencer (Applied Biosystems), and consensus sequences were generated after trimming low-quality nucleotides using CLC Main Workbench v.8.0.1 software. The sequences were submitted to BLASTn (Nucleotide Basic Local Alignment Search Tool) at the National Center for Biotechnology Information database (NCBI; http:/www.ncbi.nlm.nih.gov/Blast) for similarity analysis. Thus, the highest identity accesses (at least 95%) were selected for further phylogenetic inferences. All sequences generated were deposited to the GenBank database under the accession numbers MT135750.1–MT135757.1.

Based on the selected bacterial isolates used on the following co-cultivation experiment, four datasets were generated encountering BLASTn similarity results of isolates (*i*) Ba03, (*ii*) Ba16, (*iii*) Ba24, and (*iv*) all eight 16S rRNA partial region sequences obtained in the present study (Isolates). Individually, each dataset was aligned by the ClustalW algorithm (Thompson *et al.* 1994), incorporated in CLC Main Workbench v.8.0.1 software, and manually edited. Based on the Akaike information criterion (AIC) implemented on jModelTest v.3.5 (Posada 2008), the best-fit model of sequence evolution corresponding to each dataset was assigned as follows: TIM+I+G for Ba03, HKY+I+G for Ba16, TrN+I+G for Ba24, and TrN+G for Isolates.

Bayesian Inference (BI) phylogenetic analysis was performed in MrBayes v.3.2.6 (Ronquist and Huelsenbeck 2003) on CIPRES Science Gateway V.3.1 (www.phylo.org; Miller *et al.* 2010). The BI analysis was carried out for two independent runs with 4 chains each, and 200,000 generations with tree sampling every 100 generations. The first 25% of tree sampling was discarded as burn-in, and posterior probabilities (PP) were estimated by constructing a majority-rule consensus with the remaining trees. Trees were visualized and edited with FigTree v.1.3.1 (Rambaut 2010). *Escherichia coli* 16S rRNA partial sequence (NR024570.1) was used as an outgroup.

In vitro* co-culture of isolates with *Guadua chacoensis For the *in vitro* co-cultivation experiment, previously established *Guadua chacoensis* (Rojas Acosta) Londoño & P.M. Peterson *in vitro* cultures were used. Clumps with 3 to 6 shoots were obtained from the multiplication phase with 15 μM of 6-benzylaminopurine (BAP) (Sigma-Aldrich) and subcultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (Sigma-Aldrich) supplemented with 2 mL L^{-1} of Morel vitamins (Morel and Wetmore 1951) (PhytoTechnology Laboratories, Lenexa, KS), 30 g L^{-1} sucrose, and gelled with 2 g L^{-1} Phytagel® (Sigma-Aldrich). The pH of the culture media was adjusted to 5.8 before autoclaving (121°C and 1.3 atm) for 15 min. The cultures were kept in a growth room under controlled temperature (24°C ± 2°C) and photoperiod (16h) conditions. In order to minimize residuals effects of BAP, the cultures were maintained during 40 d in absence of plant growth regulators. Afterwards, the clumps were subcultured into test tubes containing 20 mL of MS basal culture medium, using MS medium as a control treatment (control I).

The selected bacterial isolates (Ba03, Ba16, Ba24) were cultured in a conical flask with 300 mL of liquid LB culture medium and maintained at 30°C for 24 h. These three bacterial cultures and LB medium (control II) were subjected to serial dilutions of 10^{-6}, 10^{-7}, and 10^{-8}. Then, 100 μL of each concentration of bacterial cultures and LB medium was inoculated on MS medium in the basal region of the *in vitro* clumps after 15 d of beginning subculture. These plants were subcultured 15 d before the bacterial inoculation to check the non-occurrence of alien microorganism contamination in plants' cultures during the medium transference. Growth evaluation was assessed by morphological parameters, consisting of the quantification of root number (NR) and length (LR) and shoot height (HS). Plant multiplication rate was determined by the ratio between the different numbers of shoots per clump at 30 d of co-cultivation (day 45) and the initial number of shoots (day 0) per initial number of shoots.

The experimental design was bifactorial (4 × 3) with an additional control. The first factor consisted of three bacterial isolates and LB medium as control (named control II). The

second factor was composed of three dilutions (10^{-6}, 10^{-7}, and 10^{-8}) of each level of the first factor. The additional control (control I) was the conventional *in vitro* culture in MS medium. Each experimental unit consisted of one test tube containing 20 mL of MS medium, with a clump of 3 to 6 shoots. The experiment was conducted with three replicates per treatment and was repeated three times under the same conditions. Quantitative data were submitted to analysis of variance (ANOVA) and SNK test (5%) of mean separation, according to Compton (1994), on the R platform (R Core Team 2020), and using the Agricolae package (Mendiburu 2019).

Results

Isolation and morphological characterization of endophytic bacteria Endophytic bacteria from *in vitro* cultures of *Dendrocalamus asper* and *Bambusa oldhamii* were isolated by repeated streaking-plating method, revealing consistent pure bacterial isolates. Among the bacterial isolates (Ba01-Ba32), 100% were Gram-positive, and the characterization of the colonies by color, size, elevation, border type, transparency, and structure is summarized in Table 1.

Regarding growth rate, the Ba03 isolate (*Serratia marcescens*) showed the highest average, ranging from 30 to 300 CFU mL^{-1} of medium, whereas the Ba24 isolate (*Brevibacillus parabrevis*) showed an average growth of up to 30 CFU mL^{-1} medium. The Ba16 isolate (*Bacillus subtilis*) was not possible to quantify, because the colonies growth was less than 30 CFU, so the count was disregarded.

Quantification of indole compounds The spectrophotometric method based on Salkowski's reagent suggests that all the endophytic bacterial strains were able to produce indole compounds in the growing media, ranging from 3.0 μg mL^{-1} (Ba16) to 15.73 μg mL^{-1} (Ba24), with an average of 8.41 μg mL^{-1} (Table 1). The three bacterial isolates selected for the subsequent co-cultivation experiment were representative of distinct groups, those based on the lowest- (Ba16), medium- (Ba03; 9.03 μg mL^{-1}) and highest-yield (Ba24) production of IC.

Molecular characterization and phylogenetic inferences Eight partial sequences of the 16S rRNA gene were generated, selecting bacterial isolates with the low- (Ba16, Ba18, Ba32), medium- (Ba03, Ba05, Ba21), and high-yield (Ba24, Ba29) IC production. Among those, BLASTn search revealed the greatest similarity with four genera belonging to three distinct families: *Bacillus* (Bacillaceae), *Brevibacillus* (Paenibacillaceae), and *Atlantibacter* and *Serratia* (both Enterobacteriaceae) (Table 2). Phylogenetic analysis based on the 16S rRNA partial sequences of the eight isolates resolved four main clades with high support (PP = 1), distinguishing the four genera clustered in their respective families, reinforcing BLASTn results (Fig. 1).

Revealing great identity with *Serratia* genus, the topology clustered Ba03 (medium-yield IC production) with *Serratia* accessions (PP = 0.78), forming a strongly supported clade with *S. marcescens* species (PP = 0.93) (Fig. 2). The isolate Ba16 (low-yield IC production) presented identity exclusively with *Bacillus* genus, with wide range variation of species. Although this isolate showed highest identity with *B. subtilis* (98.71%) (Table 2), it clustered with *B. subtilis* accessions with low support (PP = 0.61) (Fig. 3). The greatest similarity of Ba24 isolate (high-yield IC production) with *Brevibacillus parabrevis* (96.89%) is strongly supported (PP = 0.98) by the forming clade with such accessions (Fig. 4). Those results were considered as accurate species identification for the aforesaid bacterial isolates, supported by BLASTn search results.

In vitro* co-culture of isolates with *Guadua chacoensis Three days after inoculation, bacterial growth was observed in the bamboo rhizosphere and the surface of the culture media for all the inoculation treatments, in which no contamination with alien microorganisms along the *in vitro* multiplication phases was visually detected (Fig. 5). An overview of the effects of co-cultivation of the three bacterial isolates on the developmental parameters of *in vitro* plants of *G. chacoensis* is described in Table 3.

Regarding differences between treatments, means combining inoculum dilutions, the results showed that no bacterial treatment promoted relevant plant growth effect on the analyzed parameters, suggesting a neutral growth potential compared to both control treatments. Yet, plants co-cultivated with Ba03 isolate (*S. marcescens*) showed the shortest shoot height (3.26 cm) and root lengths (2.55 cm), and those co-cultivated with Ba24 isolate (*Br. parabrevis*) also showed shorter root lengths (3.58 cm) compared to control treatments (Table 3).

The effects of inoculum dilutions were similar to the means of treatments, showing no positive plant growth effect (highest values) when compared to both controls. However, lower dilutions rates (10^{-6} and 10^{-7}) resulted in low values in plant multiplication rate with Ba16 (*B. subtilis*) co-culture, as well as in number and root lengths of plant co-cultivated with Ba24 isolate (*Br. parabrevis*), suggesting an inhibitory effect in those parameters (Table 3).

Discussion

Manifestation of endophytic bacteria in *in vitro* plant tissue culture is commonly treated as harmful contaminants, even if this plant-association dynamic is generally considered to be present in all living forms (Turner *et al*. 2013; Orlikowska

Table 1. Morphological characterization and indole compounds (IC) production yield of bacterial isolates from *in vitro* cultures of *Dendrocalamus asper* (Schult. & Schult. f.) Backer ex K. Heyne and *Bambusa oldhamii* Munro, based on Rodina's methodology (1972)

Isolate*	Size [d]	Color [e]	Shape	Border	Elevation	Structure	IC [f] (μg mL^{-1})
Ba01	M	R/−	Rounded	Smooth	Convex	Filiform	11.10 ± 0.56
Ba02	M	R/−	Rounded	Smooth	Convex	Filiform	11.08 ± 0.10
Ba03 [b]	M	R/−	Rounded	Smooth	Convex	Filiform	9.03 ± 0.12
Ba04	L	W/+	Rounded	Smooth	Convex	Filiform	10.39 ± 0.22
Ba05	L	W/+	Rounded	Smooth	Convex	Filiform	9.68 ± 0.21
Ba06	M	W/+	Rounded	Smooth	Convex	Filiform	8.64 ± 0.39
Ba07	M	R/−	Rounded	Smooth	Convex	Filiform	11.08 ± 0.25
Ba08	M	R/−	Rounded	Smooth	Convex	Filiform	9.92 ± 0.22
Ba09	M	R/−	Rounded	Smooth	Convex	Filiform	10.43 ± 1.62
Ba10	M	R/−	Rounded	Smooth	Convex	Filiform	9.79 ± 0.59
Ba11	S	W/−	Rounded	Smooth	Convex	Filiform	4.15 ± 0.24
Ba12	M	Y/+	Rounded	Smooth	Flat	Filiform	7.68 ± 0.17
Ba13	M	R/−	Rounded	Smooth	Flat	Filiform	10.80 ± 0.36
Ba14	M	R/−	Rounded	Smooth	Flat	Filiform	9.90 ± 0.71
Ba15	S	W/+	Rounded	Corrugated	Flat	Filiform	3.98 ± 0.44
Ba16 [a]	L	W/−	Wrinkled	Irregular	Elevated	Friable	3.00 ± 0.27
Ba17	S	W/+	Rounded	Corrugated	Flat	Filiform	6.47 ± 0.78
Ba18	M	W/−	Wrinkled	Irregular	Droplet-like	Friable	3.47 ± 1.04
Ba19	S	W/+	Rounded	Smooth	Elevated	Filiform	4.25 ± 1.06
Ba20	L	W/−	Rounded	Smooth	Convex	Filiform	4.85 ± 0.27
Ba21	S	Y/+	Rounded	Smooth	Convex	Filiform	9.67 ± 0.10
Ba22	L	W/−	Rounded	Smooth	Convex	Filiform	6.36 ± 1.68
Ba23	S	Y/+	Rounded	Smooth	Convex	Filiform	11.49 ± 4.45
Ba24 [c]	S	Y/+	Rounded	Smooth	Convex	Filiform	15.73 ± 4.13
Ba25	M	W/−	Rounded	Smooth	Convex	Filiform	12.02 ± 0.45
Ba26	S	Y/−	Rounded	Smooth	Flat	Filiform	8.42 ± 0.05
Ba27	S	Y/−	Rounded	Smooth	Flat	Filiform	6.11 ± 0.56
Ba28	S	Y/+	Rounded	Smooth	Convex	Filiform	8.60 ± 1.42
Ba29	S	Y/+	Rounded	Smooth	Convex	Filiform	14.51 ± 2.27
Ba30	S	W/−	Rounded	Smooth	Convex	Filiform	3.18 ± 0.40
Ba31	S	Y/+	Rounded	Smooth	Convex	Filiform	9.88 ± 0.66
Ba32	L	W/−	Rounded	Corrugated	Elevated	Friable	3.34 ± 0.18

low-yield, [b] medium-yield, and [c] high-yield indole compounds (IC) production group; [d] size: small (S), medium (M), and large (L); [e] color/transparency: red (R), white (W), and yellow (Y); with (+) or (−) without transparency; [f] mean ± standard error of mean

et al. 2017). Some endophytes can be classified as plant growth-promoting bacteria (PGPB), *i.e.*, those capable to promote a wide range of beneficial effects on plant health and physiology in terms of growth rate, tolerance to environmental stress, and pathogen control (Kim *et al*. 2011; Glick 2012; Ramakrishna *et al*. 2019). Although PGPB increases plants' growth potential, their application in plant micropropagation systems is still rare (Abreu-Tarazi *et al*. 2010; Orlikowska *et al*. 2017; Kargapolova *et al*. 2020).

The composition of endophytic population is not necessarily plant species–specific and may be dependent on tissue and developmental stage, as well as ecological culture system and environmental conditions at the time of sampling (Moshynets *et al*. 2012; Liu *et al*. 2017; Zhang *et al*. 2019b; Zheng and Lin 2020). In total, the 32 endophytic bacteria isolated from *in vitro* plants of *B. oldhamii* and *D. asper* were capable to produce IC, group to which indole-3-acetic acid (IAA) belongs. This is an important phytohormone produced by many strains of PGPB and is known to be involved in several plant growth responses, and as a major factor for stimulation of root system development (Spaepen *et al*. 2007; Duca *et al*. 2014; Goswami *et al*. 2016).

Based on their IC production and potential use in the co-cultivation experiment, eight bacterial isolates were identified by 16s rRNA partial sequences and phylogenetic analysis, a widely used tool for identification of bacterial strains

Table 2. BLASTn results for similarity search of the 16S rRNA partial sequences generated from bacterial isolates from *in vitro* plants of *Bambusa oldhamii* (Schult. & Schult. f.) Backer ex K. Heyne and *Dendrocalamus asper* Munro

Isolate	Sequence size (pb)	GenBank accession n. [(a)]	Nearest bacterial species	GenBank accession n. (BLASTn)	Identity (%)
Ba03	1380	MT135750	*Serratia marcescens*	NR_114043.1	98.04%
Ba05	1370	MT135751	*Bacillus proteolyticus*	NR_157735.1	99.12%
Ba16	1390	MT135752	*Bacillus subtilis*	NR_102783.2	98.71%
Ba18	1395	MT135753	*Brevibacillus parabrevis*	NR_113589.1	99.28%
Ba21	1396	MT135754	*Atlantibacter hermannii*	NR_104940.1	97.64%
Ba24	1415	MT135755	*Brevibacillus parabrevis*	NR_113589.1	96.89%
Ba29	1408	MT135756	*Atlantibacter hermannii*	NR_104940.1	97.09%
Ba32	1412	MT135757	*Bacillus aerius*	NR_118439.1	99.29%

[(a)] GenBank accession number for the eight generated 16S rRNA partial sequences in the present study

(Chakraborty *et al*. 2014). Encompassing two distinct phyla, Gammaproteobacteria and Firmicutes, 6 species and 4 genera were recovered (*viz*. *Serratia*, *Atlantibacter*, *Brevibacillus*, and *Bacillus*). Those phyla are considered major groups of PGPB (Ramakrishna *et al*. 2019), being isolated as endophytes in several crops (Rojas-Tapias *et al*. 2012; Moronta-Barrios *et al*. 2018; Deng *et al*. 2019) and also in bamboo species (Han *et al*. 2009; Zhang *et al*. 2019b; Singh *et al*. 2020; Zheng and Lin 2020).

Morphological characterization seems congruent with molecular identification (Realpe *et al*. 2002), with exception of those neighboring the Enterobacteriaceae family (*S. marcescens*—Ba03; and *Atlantibacter hermanii*—Ba21 and Ba29). Those were characterized as Gram-positive instead of the Gram-negative expectation (Octavia and Lan 2014), which may be due to the unproper remotion of lipids from the bacterial cell wall, and retention of the primary dye, then causing inaccurate Gram-positive staining. It is important to note that this is the first report of *B. proteolyticus* (Ba05) and *A. hermanii* (Ba21 and Ba29) as endophytes in bamboos species.

The three isolates used in the co-cultivation experiment were taxonomic designated as *Bacillus subtilis* (Ba16), *Serratia marcescens* (Ba03), and *Brevibacillus parabrevis* (Ba24), bacterial species widely recognized as PGPB and employed in biotization/bio-priming and phytoremediation practices (Mohamed and Gomaa 2012; Almaghrabi *et al*. 2014; Mahmood *et al*. 2016; Akinrinlola *et al*. 2018). They

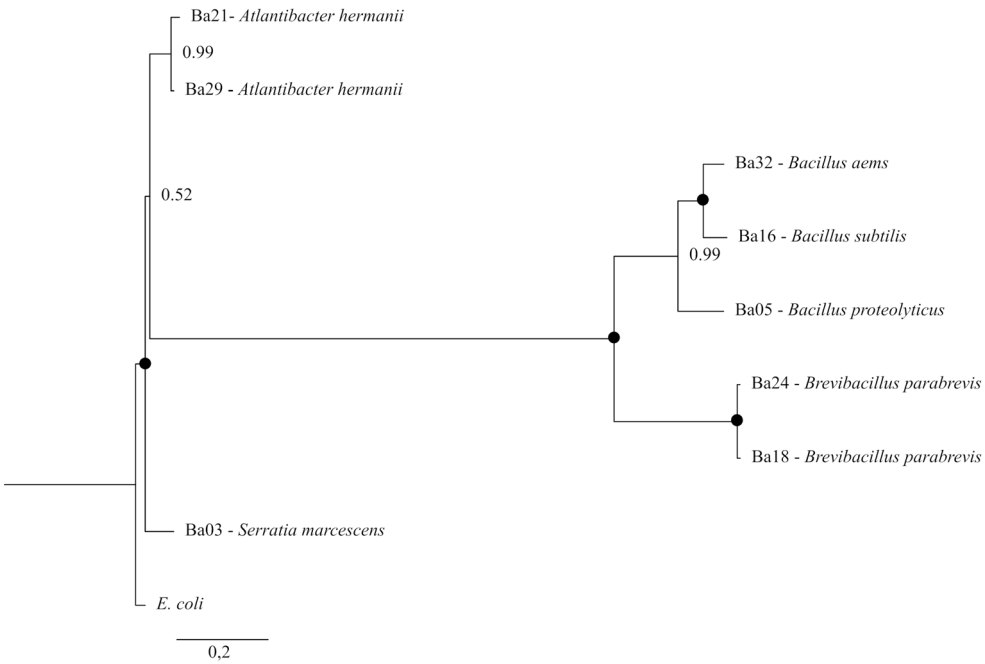

Figure 1. Bayesian inference tree based on the 16S rRNA partial sequences of the eight bacterial isolates. Clusters of genera indicate their respective families (Paenibacillaceae for *Brevibacillus*, Bacillaceae for *Bacillus*, and Enterobacteriaceae for *Serratia*). Names of each isolate were assigned after BLASTn search. *Numbers* on the nodes represent Bayesian posterior probability (PP). *Round circles* represent PP = 1.

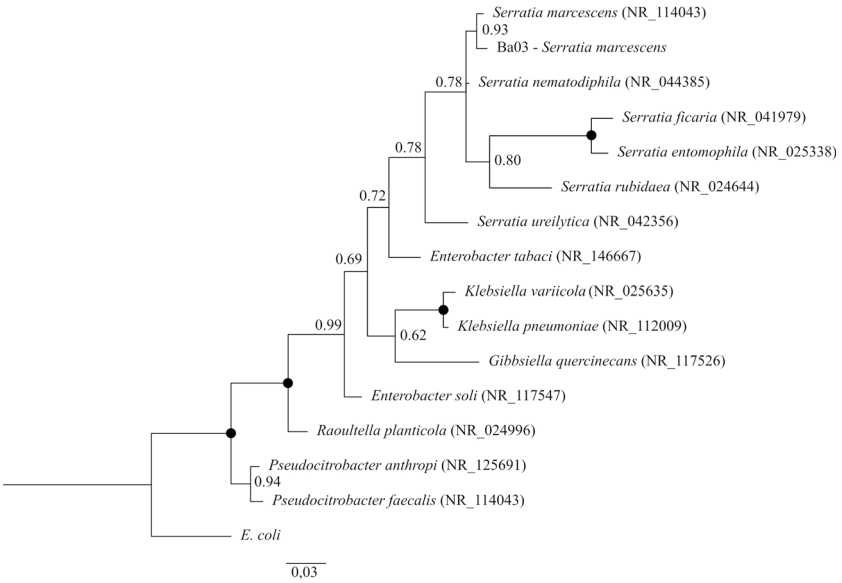

Figure 2. Bayesian inference tree based on the 16S rRNA partial sequences of Ba03—*Serratia marcescens*—after BLASTn. *Numbers* on the nodes represent Bayesian posterior probability (PP). *Round circles* represent PP = 1.

have been isolated and characterized as endophytes in several plant species, including bamboos, and have expressed plant growth potential in several different manners (Thomas and Soly 2009; Chakraborty *et al.* 2010; Moshynets *et al.* 2012; Yuan *et al.* 2015; Eevers *et al.* 2016; Asaf *et al.* 2017; Ray *et al.* 2017; Moronta-Barrios *et al.* 2018; Fancello *et al.* 2020; Pace *et al.* 2020).

Despite the remarkable positive correlation between *B. subtilis* and *S. marcescens* strains and IAA production (Almaghrabi *et al.* 2014; Goswami *et al.* 2015; Asaf *et al.* 2017), the two corresponding isolates, Ba16 and Ba03, were placed within the low- and medium-yield IC production categories, respectively. It is worthy to mention that the employed spectrophotometric method using Salkowski reagent is a widely used but simple technique, since it reacts with indole derivatives others than specifically IAA, misleading general quantification of IAA produced by PGPB (Gutierrez *et al.* 2009; Goswami *et al.* 2015; Patel *et al.* 2015). Bacterial IAA biosynthesis seems to be regulated by growth stage and culture media conditions, such as pH, carbon and oxygen sources, temperature, and environmental stress levels affecting bacterial colonies, resulting in a yield variation among strains (Spaepen *et al.* 2007; Swain and Ray 2008).

Several *in vivo* and *in vitro* assays revealed distinct effects on PGPB IAA producers in plant developmental parameters, those being positive, neutral, or negative (Ulrich *et al.* 2008;

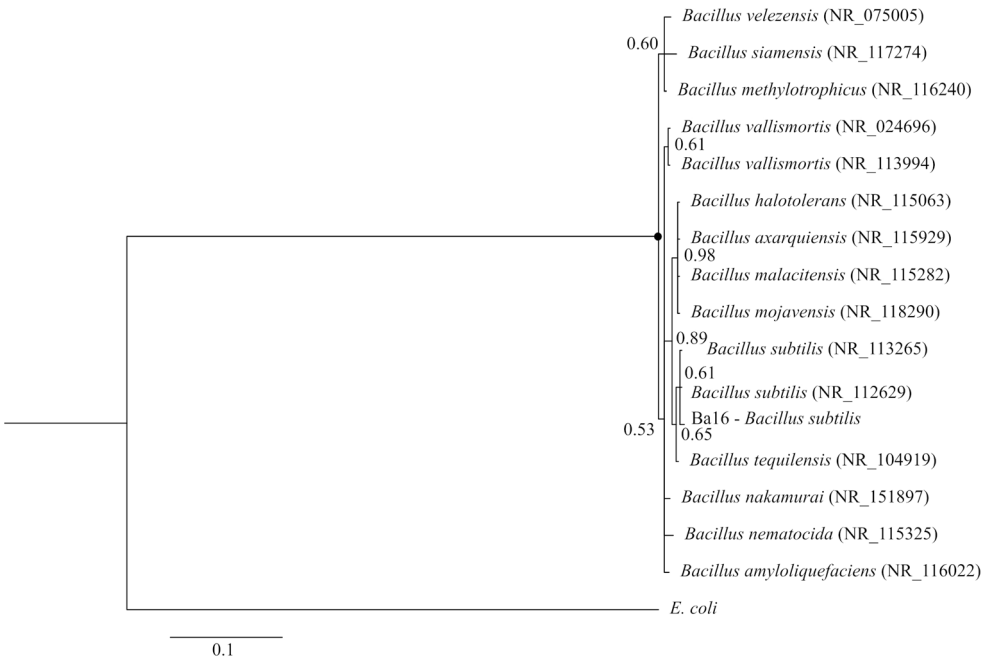

Figure 3. Bayesian inference tree based on the 16S rRNA partial sequences of Ba16—*Bacillus subtilis*—after BLASTn. *Numbers* on the nodes represent Bayesian posterior probability (PP). *Round circles* represent PP = 1.

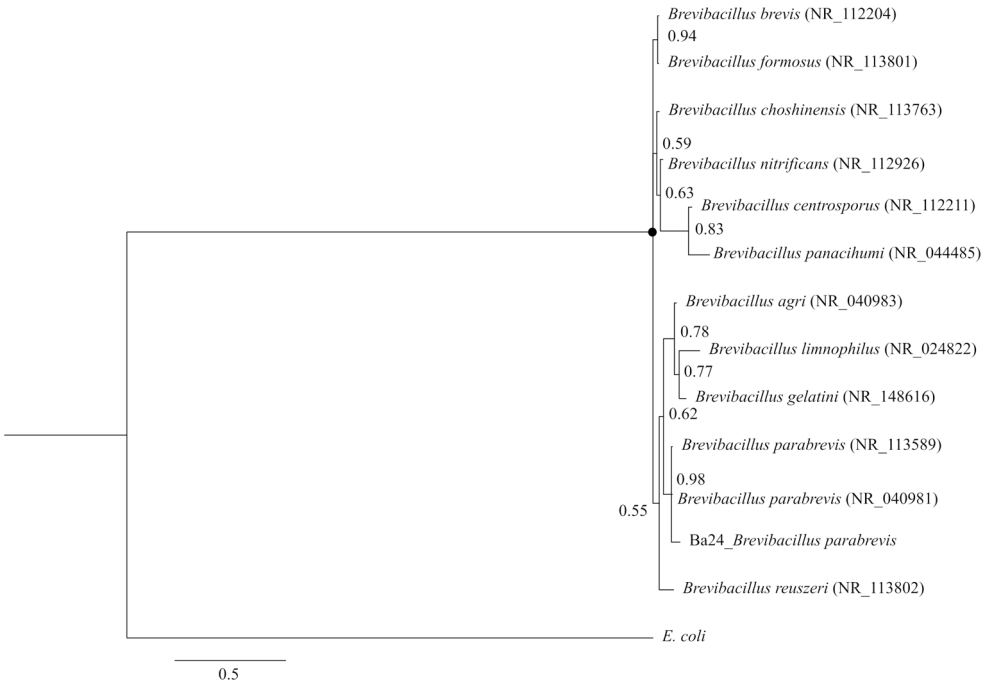

Figure 4. Bayesian inference tree based on the 16S rRNA partial sequences of Ba24—*Brevibacilus parabrevis*—after BLASTn. *Numbers* on the nodes represent Bayesian posterior probability (PP). *Round circles* represent PP = 1.

Smyth *et al*. 2011; Arslan and Akkaya 2020; Kargapolova *et al*. 2020; Pace *et al*. 2020). Bacterial IAA producers interact with plants in a spatiotemporal manner, in which the responses are primarily dependent on plant endogenous IAA content and sensitivity to exogenous IAA (Spaepen *et al*. 2007; Gutierrez *et al*. 2009). The growth-promotion stimulus of the three bacterial inoculations, and their dilution levels, in co-cultivation with *in vitro* plants of *G. chacoensis*, was

Table 3. Multiplication rate, number and height of shoots, and number and length of roots of *Guadua chacoensis* (Rojas Acosta) Londoño *in vitro* plants co-cultivated with bacterial isolates

Inoculum	Dilution	Shoots				Roots			
		Multiplication rate		Height (cm)		Number		Length (cm)	
MS (control I)	-	2.25 ± 0.24	A	3.91 ± 0.15	A	4.59 ± 0.33	A	6.38 ± 0.29	A
LB (control II)	10^{-6}	2.06 ± 0.45	a	3.36 ± 0.28	a	2.78 ± 0.68	a	5.54 ± 0.57	a
	10^{-7}	1.92 ± 0.23	a	3.70 ± 0.20	a	4.28 ± 2.11	a	5.78 ± 0.74	a
	10^{-8}	1.81 ± 0.26	a	4.01 ± 0.18	a	3.11 ± 0.40	a	5.91 ± 0.96	a
	Mean	1.93 ± 0.07	A	3.69 ± 0.19	A	3.39 ± 0.45	A	5.74 ± 0.11	A
S. marcescens (Ba03)	10^{-6}	2.26 ± 0.38	a	3.28 ± 0.11	a	4.22 ± 1.18	a	2.56 ± 0.47	a
	10^{-7}	1.94 ± 0.21	a	3.15 ± 0.06	a	2.56 ± 0.29	a	2.54 ± 0.17	a
	10^{-8}	2.40 ± 0.34	a	3.35 ± 0.15	a	2.89 ± 0.73	a	2.55 ± 0.62	a
	Mean	2.20 ± 0.13	A	3.26 ± 0.06	B	3.22 ± 0.51	A	2.55 ± 0.01	B
B. subutilis (Ba16)	10^{-6}	1.82 ± 0.02	b	3.80 ± 0.19	a	3.78 ± 1.37	a	5.88 ± 1.84	a
	10^{-7}	1.95 ± 0.10	b	3.85 ± 0.15	a	3.67 ± 0.84	a	5.22 ± 1.28	a
	10^{-8}	2.30 ± 0.07	a	3.90 ± 0.10	a	4.33 ± 0.69	a	6.35 ± 0.54	a
	Mean	2.02 ± 0.14	A	3.85 ± 0.03	A	3.93 ± 0.21	A	5.82 ± 0.33	A
Br. parabrevis (Ba24)	10^{-6}	2.06 ± 0.49	a	3.72 ± 0.45	a	2.33 ± 0.51	b	1.68 ± 0.63	b
	10^{-7}	1.72 ± 0.11	a	3.74 ± 0.26	a	1.89 ± 0.48	b	3.25 ± 0.49	ab
	10^{-8}	2.16 ± 0.29	a	3.78 ± 0.11	a	7.22 ± 1.78	a	5.81 ± 1.17	a
	Mean	1.98 ± 0.13	A	3.75 ± 0.02	A	3.81 ± 1.71	A	3.58 ± 1.20	B

Uppercase and *lowercase* letters in the *columns* do not differ from each other by SNK test at 5% probability, regarding combined treatments and dilutions, respectively. *MS*, Murashige and Skoog medium; *LB*, Luria Bertani solid medium

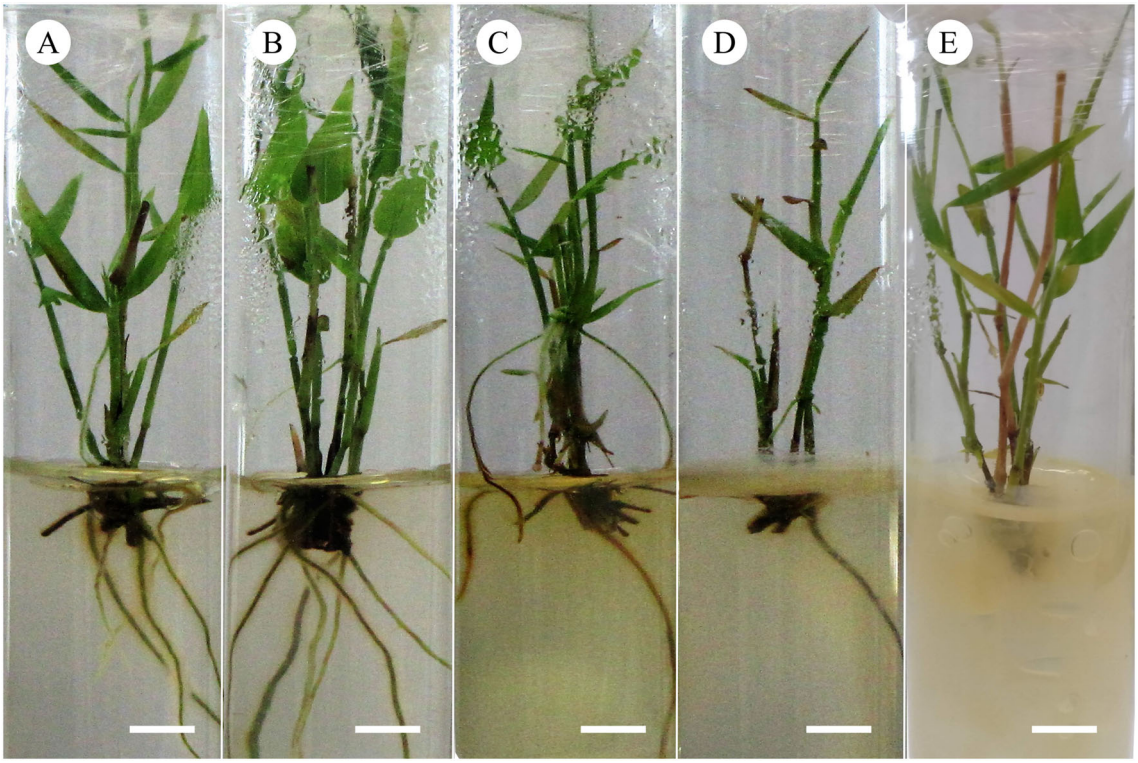

Figure 5. *Guadua chacoensis in vitro* culture after 30 d of bacterial inoculation. (*A*) Control I on Murashige & Skoog (MS) medium without bacterial inoculation; (*B*) Control II on Luria Bertani (LB) medium without bacterial inoculation; (*C*) Ba03—*Serratia marcescens*; (*D*) Ba16—*Bacillus subtilis*; (*E*) Ba24—*Brevibacilus parabrevis.*

remarkably neutral in most analyzed plant developmental features, showing an even inhibitory effect of lowest dilution levels in multiplication rate, root number, and root length. Such a result suggests that the optimal inoculum level under a plant-bacterial co-cultivation assay is crucial to accurate predictions of plant response to exogenous IAA focusing on the potential use of PGPB (Duca *et al*. 2014; Arslan and Akkaya 2020).

Endophytic microorganism manifestation in presumed axenic *in vitro* cultures has been questioned since they could be in a latent or in a permanent manner in those cultures (Almeida *et al*. 2009; Abreu-Tarazi *et al*. 2010). Thus, regarding the wide beneficial plant-bacterial interactions and the great bamboo biodiversity, a deeper investigation of bamboo endophytes would expand our knowledge on the benefits of those bacterial communities and their potential as plant growth promoters in this recognized multifunctional plant (Moshynets *et al*. 2012; Ramakrishna *et al*. 2019).

Conclusion

The first isolation of endophytic bacteria from *in vitro* cultures of *Dendrocalamus asper* and *Bambusa oldhamii* was performed, as well as the first *in vitro* co-cultivation experiment with potential PGPB and a bamboo species, *G. chacoensis*. The bacterial collection was composed of 32 isolates capable to produce IC, then showing growth-promotion potential. In general, the results suggest that endophyte inoculation did not cause harmful effects on developmental parameters in *in vitro* plants of *G. chacoensis*. Therefore, the reported possibility of co-existence of endophytic bacteria in *in vitro* systems without major damages compromising plant development would contribute to enhancement of bamboo micropropagation methods and the large-scale production of plantlets.

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