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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Assessing the functional diversity of rhizobacteria from cacao by partitioning root and shoot biomasses

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Abstract

Plant–microbe interactions are critical for the sustainability of agricultural production. In this study, our aims were to characterize the genetic and functional diversity of the culturable bacterial community associated with the cacao rhizosphere and access their potential for growth promotion of cacao seedling. Culture-dependent and molecular methods were used to characterize the population densities and diversity of bacterial communities from soil and cacao plants at two locations and two plant ages. A total of 63 strains were identified through *hsp60* sequencing. *Pseudomonas* and *Enterobacter* were the most abundant genera in association with the cacao rhizosphere, whereas *Bacillus* was more numerous in soil. Parameters of seedling growth promotion were evaluated 60 days after inoculation of seeds, with partition of the assessments into root and shoot weight. Each isolate showed beneficial, neutral or deleterious effects on plant growth, depending on the isolate and on the parts of plant assessed. Interestingly, although an apparent overall decrease in total biomass of seedlings (roots + shoots dry matters) was observed for the majority of isolates (89%), 94% of all isolates, in fact, revealed an increase in plant roots/shoots dry biomass ratio. Despite that part of the isolates (35%) appeared to significantly decrease plant height, and that 65% did not influence plant height (neutral effect), 18 had significantly increased root dry biomass; nevertheless, seven of these root growth-increasing isolates simultaneously decreased shoots-related growth parameters. The results of this study evidentiated the functional diversity of culturable cacao rhizobacteria and how the partitioning of roots and shoots in the assessment of plant growth parameters could reveal the biotechnological potential of these isolates for promoting growth of clones for rehabilitation of commercial cacao plantations.

Key Points

- *The most common culturable bacteria in cacao roots were Pseudomonas and Enterobacter*
- *Most culturable bacteria from cacao roots increased the root/shoot ratio*
- *Roots and shoots should be examined separately to detect cacao beneficial bacteria*

Keywords Cocoa · Root growth promotion · Microbial diversity · Root/shoots ratio · Phytohormones

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Introduction

Cacao (*Theobroma cacao* L.) is a perennial, arboreal plant, typical of tropical climates and native to the Amazon rainforest (Bartley 2005). This crop is of worldwide relevance, since its seeds ("nuts" or "almonds") provide the raw material for chocolate, food, and cosmetic industries. The market of cocoa (the main product from cacao plants) has an estimated value of 8 to 10 billion dollars, and Brazil is the sixth largest producer (Laliberté et al. 2012). A drastic decline in the Brazilian cocoa production began in 1989 with the

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outbreak of the witches' broom disease in Bahia, coupled with a large fall in international prices of this commodity (Pereira et al. 1996; Anderbrhan et al. 1999). Other pathogens such as those from the oomycete genus *Phytophthora* have also contributed to this low cocoa production context (Hanada et al. 2009). Cacao also bears a great social and ecological value for Brazil, due to the employment of the local labor force and its growth in understory, thus serving as an important component of management strategies both for biodiversity conservation in rain forests (Schroth et al. 2011; Sambuichi et al. 2012) and for improved C storage at landscape level (Schroth et al. 2015). Therefore, the development of strategies to increase the overall productivity of cacao and to rehabilitate commercial plantations are yet required (Medeiros et al. 2010; Wickramasuriya and Dunwell 2018).

To assure to farmers a proper provision of seedlings from improved genotypes developed by breeding programs (Sodré et al. (2012), a nursery period is part of the cacao production system. Since this period is an onerous, labor-intensive phase, any strategy capable of shortening the seedlings production time while assuring their nutritional and phytosanitary quality is desired (Sodré et al. 2012). Soil microbiomes are among the world's most complex communities, where plant–microbe interactions are critical for the sustainability of agricultural production (Beneduzi et al. 2012; Singh 2013; Vacheron et al. 2013; Vejan et al. 2016). Among the vast array of microorganisms present in the soil/rhizosphere, the plant growth–promoting rhizobacteria (PGPR) are of special agronomic interest, since they can help on the growth and development of a range of annual (Chen et al. 2000; Santos et al. 2020) and perennial plant species (Vonderwell et al. 2001; Mafia et al. 2009). Hence, the use of selected PGPR in cacao nurseries can generate benefits by increasing shoot and/or roots dry masses, which can result in a reduced plant cycle and/or increases in health and/or productivity (Mafia et al. 2009; Vacheron et al. 2013; Santos et al. 2020).

Growth induced by rhizobacteria is mainly due to activities related to phosphate solubilization, atmospheric N fixation, improved nutrient availability, production of plant growth regulators (phytohormones) and other secondary metabolites (e.g., siderophores, β-1,3 glucanases, chitinases and antibiotics), inhibition of deleterious microorganisms, and induction of systemic resistance in plants (Sturz et al. 2000; Ramamoorthy et al. 2001; Zehnder et al. 2001; Lodewyckx et al. 2002; Koening et al. 2002; Pidello 2003). Microbial characterization at a minimally proper taxonomical levels is relevant, as phylogenetic signals are a first-tier indication of functional properties (Morrissey et al. 2016). Among the various genera of possibly beneficial bacteria present in soil, *Bacillus*, *Enterobacter*, and *Pseudomonas* have been most widely found (Adesemoye et al. 2008; León et al. 2009; Singh 2013; Park et al. 2015). These bacteria have highly desirable characteristics, such as the ability to produce secondary metabolites, great nutritional versatility, ability to grow in a variety of environments, and high potential for root colonization (Chanway et al. 2000; Aagot et al. 2001; Singh 2013; Vejan et al. 2016). Also, members of these genera have shown indirect effects in plant resistance by both the induced systemic resistance (ISR) and the systemic acquired resistance (SAR) pathways (Beneduzi et al. 2012).

Diversity, phylogenetic, taxonomic, and functional studies of rhizobacteria can provide relevant information for further isolation and selection of strains that can aid in maintenance of soil fertility and in upgrades on plant growth and development (Morrissey et al. 2016; Vejan et al. 2016). Beyond the relevance of the 16S rRNA gene for diversity and ecological investigations (Øvreås and Curtis 2011), other genes have also been used for taxonomic and phylogenetic studies of microorganisms (Dahllof et al. 2000; Souza et al. 2003). For instance, heat shock proteins genes (*hsp*) have allowed the identification of phylogenetically close species (Karlin and Brocchieri 2000; Hu et al. 2018), as it is usually found as a single copy per cell and tends to display a more accelerated evolution than 16S rDNA. In microbial diversity studies, both culture-independent and -dependent systems can be used to study the composition of a given bacterial community. While the former has a much larger capability of tackling a closer-to-real microdiversity, due to the astonishing informational reach provided by the high-throughput next-generation sequencing (NGS) protocols (e.g., Rappé and Giovannoni 2003; Rastogi et al. 2013; Beckers et al. 2016; Lay et al. 2018), the latter allows the constitution of collections of isolates prone to physiological and functional characterizations with potential biotechnological exploitation (Schulz et al. 2002; Daniel 2004; Mosa et al. 2016).

Studies on the interactions of PGPR and plants tend to target the isolation, identification, and characterization of culturable microbes for specific purposes (Vejan et al. 2016), since culture-dependent methods provide the opportunity for further biotechnological studies and bioproducts development. Considering the approach of exploring associated microbial communities, surprisingly, there are very few reports on cacao–PGPR systems, both in terms of basic descriptive diversity and interactions, and in the potential for biotechnological applications for agriculture and industry. As pointed out above, healthy seedlings growth and mass production compose the major strategy for distribution of genetically improved clones to farmers, aiming to install new productive and disease-resistant cacao plantations. In the present work, the structural and functional diversity of culture-dependent bacterial community associated with the cacao rhizosphere were investigated, considering plantations of different ages and localities. We performed isolation, quantification, and taxonomic identification of culturable rhizobacteria, also providing a genetic, biochemical, and

physiological characterization of 63 isolates, with emphasis on their ability to promote plant growth. A key aspect of this work is related to a proper assessment of biomass parameters for detection of growth promotion or deleterious effects on cacao seedlings.

Materials and methods

Quantification and isolation of rhizobacteria from cacao

Soil and cacao root samples from the rhizosphere (limited by the vertical projection of the plants canopy area) were collected from the Institute Biofactory of Cacao (IBC) (− 14° 44′ 54.2832″ S, − 39° 4′ 1.6644″ W) and the Executive Committee for the Cacao Crop Plan (CEPLAC) (− 14° 46′ 51.0636″ S, − 39° 13′ 16.5648″ W). Both institutes are located in the municipality of Ilhéus, Bahia, Brazil; this location has a humid tropical climate, with annual temperatures ranging from 22 to 30 °C on average (a warmer period from october to march, and a cooler one from april to september) and an average annual rainfall of ~ 1325 mm (https://pt.climate-data.org/america-do-sul/brasil/bahia/ilheus-4467/). In each area (IBC and CEPLAC), the samples were collected in the month of May (average temperature of 26 °C and rainfall of ~ 113 mm) from two types of cacao plantations: "old" cacao, with more than 3 years of planting and "new" cacao, with up to 3 years of planting. Thus, a total of four collection sites (Old IBC, New IBC, Old CEPLAC, New CEPLAC) were defined, based on two grouping criteria, i.e., site location and plant age. For all collection sites, the plants used were from *T. cacao* var. *comum*. At each site, soil and root sub-samples were collected from 6 randomly selected plants. The samples were mixed to form a composite sample per treatment (site). The chemical and physical characteristics of the soil where the samples were collected from are presented in Suplementary Table S1.

The quantification of bacterial population was determined in 10 g of each sample by the ten-fold serial dilution method, followed by plating in culture medium, and colony counting. Three categories were assessed: total bacteria, *Pseudomonas* and *Bacillus*, with the latter two representing the most likely abundant Gram-negative and Gram-positive representatives, respectively (Raaijmakers et al. 2010). Three replicates were plated for each dilution (10^{-1} to 10^{-5}) from each soil and roots samples, in a total of 120 plates (3 plates × 4 sites × 2 samples × 5 dilutions). The nutrient agar medium (Levine 1954) was used with cycloheximide (10 mg L^{-1}) to determine the population densities of total bacteria. Selective NAA medium (composition described in Aagot et al. 2001) with 50 mg of casamino acids (BD Difco™, Franklin Lakes, NJ, USA) was used for the quantification of *Pseudomonas*, and the selective ATCC 573 medium ($(NH_4)_2SO_4$ 1.3 g; KH_2PO_4 0.37 g; $MgSO_4.7H_2O$ 0.25 g; $CaCl_2.2H_2O$ 0.07 g; $FeCl_3$ 0.02 g; yeast extract 1.0 g, agar 25 g, distilled water up to 1000 mL and cycloheximide 10 mg L^{-1}) was employed for the quantification of *Bacillus*. Prior to plating on ATCC 573 medium, the dilutions of soil and roots samples were incubated at 80 °C for 10 min in a water bath (Sneath 1986). The plates were incubated at room temperature (RT; 25 ± 2 °C) and bacterial populations were evaluated after 36–48 h. The statistical program SISVAR (Ferreira 2011) was used for the analysis of variance (ANOVA) and the comparison of means by the Tukey test ($P < 0.05$). To analyze the differences between bacterial populations on roots and soil in old and new cacao plantations, the *t*-test was used at $P < 0.05$. This work was repeated under the same experimental conditions and the statistical analysis was performed separately.

A sub-sample of the total culturable rhizobacteria, totaling up 59 isolates from all four collection sites, were randomly selected for further experiments. Four extra endophytic isolates (labeled as ALB–353, –369, –684, and –629) were obtained from healthy cacao plantations at the Center for Cacao Research–MARS Almirante Cacau farm to serve as comparison references. Therefore, the total number of isolates assessed in this study was 63 (see next section).

Genetic characterization of isolates

For the extraction of genomic DNA, the 63 rhizobacterial isolates described above were grown in tryptic soy agar medium (TSA: peptone 5 g, tryptone 15 g, NaCl 5 g, agar 15 g, distilled water up to 1000 mL). After 24 h, colonies from each isolate were transferred to 1-mL microtubes containing 100 µl of cell lysis buffer (0.05 M NaOH + 0.25% sodium dodecyl sulfate—SDS) and incubated in a water bath for 15 min at 100 °C. Then, the tubes containing the samples were centrifuged at 10,000 rpm for 1 min in a microcentrifuge (Eppendorf™ 5417R, Hamburg, Germany) and the DNA in the supernatant was collected, diluted 20-fold in Milli-Q® (Merck, Rahway, NJ, USA) water and stored at − 20 °C.

Amplification of the *hsp60* gene fragment for molecular identification was performed with primers HSP60F (5′-GGTAGAAGAAGGCGTGGTTGC-3′) and HSP60R (5′-ATGCATTCGGTGGTGATCATCAG-3′) according to the methodology of Roggenkamp et al. (2004). The amplified products were separated in a low melting point agarose gel (NuSieve™, Lonza Bioscience, Walkersville, MD, USA) at a 1.5% concentration. The bands were cut from the gel, frozen at − 80 °C for 1 h and microcentrifuged for 15 min at 14,000 rpm. The supernatant was used directly for sequencing with the primers previously used for amplification. Sequencing was done with the BigDye Dideoxy Terminator Sequencing kit (Applied Biosystems™, Waltham, MA,

USA) according to the manufacturer's recommendations. After the sequencing reaction, the samples were precipitated with 20 µl of 65% isopropanol and remained in the dark at RT for 15 min. The 96-well plates containing the reactions were centrifuged for 40 min at 4000 rpm. Supernatants were discarded, the plate was inverted onto paper towel for drying, and 100 µl of 60% ethanol was added and centrifuged at 4 krpm for 8 min. Ethanol was discarded and the plate was centrifuged onto the paper towel at 700 rpm for 10 s to remove ethanol excess. After drying at RT for 15 min, 10 µl of formamide was added to each sample; the plate was placed in the thermal cycler for denaturation at 94 °C for 3 min. After this, the samples in the plate were subjected to the ABI PRISM 3100® Genetic Analyzer Automatic DNA Sequencer (Applied Biosystems™, Waltham, MA, USA).

The program BioEdit version 5.0.9 (Hall 1999) was used to join the sequences from the forward and reverse primers. The alignments of the 310-bp *hsp60* sequences were done with the MEGA program, version 3.1 (Kumar et al. 2004). The BLAST algorithm (Altschul et al. 1997) was used to compare the sequences of each sequenced isolate with those of type strains of species deposited in public databases.

PCR-RAPD of isolated rhizobacteria

Out of the 63 culturable isolates from cacao under study, six yielded amplicons with different sizes and sequence identification (see "Results" for details) and were not included in this polymerase chain reaction–random amplified polymorphic DNA (PCR-RAPD) analysis. Hence, the 57 isolates with 310-bp *hsp60*-specific sequenced amplicons (including the four ALB-n references, see above) were assessed in their genetic diversity as follows: DNA samples from each isolate were extracted as previously described and subjected to the PCR-RAPD technique, according to the methodology of Keel et al. (1996), using three decamer primers (D7, M12, and M13) to generate the isolate-specific patterns of amplified DNA. Amplification reactions were subjected to agarose gel electrophoresis, and the presence/absence of bands generated by RAPD primers were converted into a binary data matrix (1 for presence and 0 for absence of a particular band size in the gel), which was used to calculate the Jaccard's coefficient of similarity. Neighbor-joining clustering analysis was performed with the FreeTree® program (Hampl et al. 2001). The reliability of the dendrogram was assessed through bootstrap analysis with 1000 replicates and edition and visualization were done in TreeView® (Page 1996).

Physiological characterization of the rhizobacterial isolates

In vitro tests were performed for detection of specific metabolites, with 4 replicates per isolate. The methodology described by Renwick et al. (1991) was used to determine the production of extracellular enzymes (chitinase, cellulase, and xylanase). The production of indoleacetic acid (auxin) was evaluated using the qualitative technique of Bric et al. (1991), and the ability of phosphate solubilization was determined according to the method proposed by Katznelson and Bose (1959). All these methods were based on the detection of halos around the colony, according to the specific media composition and compounds added to the medium: a clear halo for inorganic phosphate (PhS) solubilization, a hyaline halo for cellulase (Cel) and chitinase (Chit) activity, an orange halo for xylanase (Xyl) activity, and a reddish halo in the nitrocellulose membranes for indoleacetic acid (IAA) production.

Growth promotion in cacao seedlings

A completely randomized experiment with five replicates per isolate was conducted in a greenhouse, in non-sterile soil to evaluate the potential of the 63 rhizobacteria in promoting growth of cacao seedlings (in a total of 64 treatments, including the control). The isolates were cultured in TSA medium at RT for 48 h. Cacao seeds of variety (*comum*) were collected from trees growing in an area which was not sampled for bacterial isolation. Prior to bacterial application, the seed peel was carefully removed with a scalpel and surface disinfestation was performed (10 min in 2.5% sodium hypochlorite followed by 3 washes with sterile distilled water). The seeds were then submerged in each bacterial suspension $OD_{600} = 0.1$ for 30 min and seeded in plastic pots containing 500 mL of non-sterile soil from the CEPLAC site. The control treatment consisted of seeds immersed in sterilized distilled water for 30 min. This incubation time for the seed microbiolization lies in the middle of a range of other times previously reported (Leite et al. 2013; Falcão et al. 2014).

The seedlings were kept in a greenhouse and collected 60 days after sowing; the nutritional conditions of the soil used were considered adequate, so that no fertilization or pesticide application were employed; the use of non-sterile soil aimed at providing microbial communities closer to field conditions. The number of leaves, plant height, shoot dry mass, and root dry mass were evaluated. The dry biomass was determined after washing the roots in running water and separating them from the aerial parts; the samples were then placed in a drying oven with forced ventilation at 70 °C until reaching a constant mass. The data were analyzed by the SISVAR statistical program (Ferreira 2011), and the usual assumptions of analysis of variance (ANOVA) were met. The ANOVA was performed and the means were compared by the Scott-Knott test at 5% probability. For analytical, quantification and comparison purposes, groups of isolates based on the statistical significance obtained from the

Scott-Knott test were formed for each parameter addressed: three significantly different groups of isolates (A, B, and C) were formed for plant height, two (A and B) for shoots dry matter, and three (A, B, and C) for roots dry matter; furthermore, a non-parametric Kruskal–Wallis ranking test was performed for all isolates plus the control for the values of total plant dry biomasses (roots + shoots) and the roots/shoots (R/S) ratio (see Results).

Results

Population densities of bacterial groups in the cacao rhizosphere

Population densities were determined for three culturable groups: total bacteria, *Pseudomonas*, and *Bacillus* (Table 1). These two genera were chosen because (i) they are frequently among the most abundant representatives of the Gram-positive and -negative genera in rhizospheres (when considering results from both culture-dependent and independent studies), (ii) they show a remarkable metabolic versatility and ability to dwell in a variety of ecosystems and niches, and (iii) they are widely studied for an array of anthropogenic purposes (Raaijmakers et al. 2010). Chemical (including pH, macro-, and micronutrients) and physical (silt, clay, and sand) characteristics were obtained for the four collection sites (Supplementary Table S1). The three studied groups showed populational densities that were significantly different ($P < 0.05$) between roots and soils of cacao (Table 1 and Supplementary Table S2). Total bacteria presented 2.1 to 7.9 times higher densities in roots than in soils, with a significant average difference of 4.8 times more cells for roots (Table 1 and Supplementary Table S2). Despite that only one site showed significant difference between soil and roots for total bacteria (Old CEPLAC) and for *Pseudomonas* (New IBC), the trend of higher populational densities for roots in both groups was clear (Table 1 and Supplementary Table S2). Contrariwise, the four collecting sites showed a tendency for higher populations of *Bacillus* in soils than in roots, with a significant difference between their overall means. The results also indicated that, while *Pseudomonas* presented similar average levels of occurrence within the total bacterial population for both roots and soil (ranging from 1.10 to 1.73), *Bacillus* was remarkably more represented in the total culturable bacterial community of soil than in the roots of cacao, at an average rate of 10 times more cells for the former (Table 1). In general, *Bacillus* was found to be significantly higher than *Pseudomonas* in soil and root samples from plantations older than 3 years, whereas for younger crops this difference was not verified (Table 1 and Supplementary Table S2). The number of *Bacillus* present in the CEPLAC samples was significantly higher than in the IBC samples ($P < 0.05$) (Table 1 and Supplementary Table S2). Physico-chemical properties of the soils from the four sampling sites showed that Al was absent in CEPLAC sites; moreover, Ca, K, Mg, Zn, and Mn levels were higher, as well as the silt fraction, and P levels were lower (Supplementary Table S1).

Genetic characterization and diversity of isolates

In order to qualitatively characterize the bacterial communities found in the rhizosphere environment of the cacao sites, culture-dependent methods were employed. Out of the culturable bacteria grown in nutrient agar medium, from the four collection sites, 59 morphologically distinct colonies were randomly selected (four extra isolates were added as reference—see "Methods"). Then, each of the 63 culturable isolates was identified at the genus level by sequencing of

Table 1 Bacterial population densities in cacao rhizosphere and soil samples

Sample site [a]	CFU.g^{-1} × 10^{7} [b]						% [c]			
	Total bacteria		*Pseudomonas*		*Bacillus*		*Pseudomonas*		*Bacillus*	
	Roots	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots	Soil
Old IBC	5.3 a	2.5 a	0.1 b	0.01 b	0.36 a	0.58 a	1.92	0.41	6.75	23.25
New IBC	82.3 a	19.0 a	1.94 a	0.02 b	0.58 a	1.25 a	2.31	0.09	0.70	6.57
Old CEPLAC	121 a	15.3 b	1.52 a	0.43 a	1.84 a	7.73 a	1.26	2.79	1.53	50.67
New CEPLAC	70.7 a	21.0 a	1.02 a	0.24 a	0.68 b	4.43 a	1.45	1.13	0.97	21.11
Means	69.8 a	14.4 b	1.14 a	0.17 b	0.86 b	3.49 a	1.73	1.10	2.49	25.40

[a]Samples collected at IBC and CEPLAC on cacao plantations over 3 years old (old) and less than 3 years old (new)

[b]Data in the table correspond to number of colony-forming units (CFU) per gram (g) of roots or soil, times 10 to the seventh power, and are means of three replicates (plates) per sample. The experiment was fully repeated once, and provided very similar results. Means followed by different letters are significantly different according to Tukey's test ($P < 0.05$). Comparisons should be done within each group of bacteria and between roots and soil only

[c]Percentage relative to the population of total bacteria

the *hsp60* gene. Sequenced 310-bp fragments aligned with database entries showed that the isolates belonged in the *Enterobacteriaceae* (*Enterobacter*, *Klebsiella*, *Pantoea*, and *Serratia* species, with a total of 34 isolates) and *Pseudomonadaceae* (*Pseudomonas* species with 18 isolates) families (Table 2). The two most common genera found in the cacao rhizosphere were *Pseudomonas* and *Enterobacter*, with 23 and 15 isolates, respectively (Table 2). Interestingly, the sequencing data also identified six isolates belonging in species of the *Bacillaceae* (three isolates of *Bacillus*) and *Flavobacteriaceae* (three isolates of *Chryseobacterium*) families; however, the aligned genes retrieved from the database were different from *hsp60*, revealing non-specific amplification by the primers used in this study (Table 2). Three of these non-expected fragments (isolates 110, 124, and 133) were 375-bp long that showed 95% identity to a "hypothetical protein" of bacteria from the *Chryseobacterium* genus (*Flavobacteriaceae*), whereas the other three non-specific amplicons of ˜590 bp (isolates 90, 97, and 142) were the *sucA* gene encoding the "E1 component of

Table 2 Identification of rhizobacterial isolates by sequencing a *hsp60* gene fragment and other unrelated gene fragments amplified by PCR [a]

Isolates	Gene region	Length (bp)	Accession numbers in this study [b]	Closest match BLASTN		
				Identity (%)	Accession number [c]	Species (type material)
ALB684, ALB629	*hsp60*	303	MH7 81,969, – 1970	99.26	AJ543908.1	*Enterobacter hormaechei* subsp. *steigerwaltii*
56			– 81,971	100.0		
20			– 81,982	95.59		
22			– 81,983	95.22		
3, 51			– 81,972, – 81,981	96.70	AJ417141.1	*Enterobacter asburiae*
11, 49, 24			– 81,973, – 81,974, – 81,975	97.36		
64, 65, 54, 59, 57			– 81,977, – 81,976, – 81,978, – 81,979,, – 81,980	97.03		
7			– 81,984	97.35	CP010523.2 CP084787.1	*Klebsiella variicola K quasipneumoniae*
12, 63, 103, 17			– 81,985, – 81,986, – 81,987, – 81,988	99.67		
114			– 81,989	98.68		
40			– 81,990	98.34		
85, ALB353, 13		309	– 81,991, – 81,992, – 81,993	98.38	LC007455.1	*Pantoea dispersa*
130, 102, 2, ALB369, 141		303	– 81,994, – 81,995, – 81,996, – 81,997, – 81,998	99.01	CP041233.1/ CP016948.1	*Serratia marcescens* subsp. *marcescens* / *S. surfactantfaciens*
62, 61			– 81,999, – 82,000	99.34	CP041233.1	*S. marcescens* subsp. *marcescens*
84, 44			– 82,001, – 82,002	98.68		
90	*sucA*	593	– 82,026	97.30	CP020754.1	*Bacillus thuringiensis*
97			– 82,027	95.62	CP007666.1	*B. anthracis*
142		463	– 82,028	95.90		
124, 133, 110	Hyp [c]	375	– 82,029, – 82,030, – 82,031	94.27	LR134289.1	*Chryseobacterium gleum*
21, 47, 58, 15	*hsp60*	303	– 82,003, – 82,022, – 82,023, – 82,024	93.65	HG322950.1	*Pseudomonas knackmussii*
4			– 82,008	92.98		
1, 35, 39, 67, 5, 23, 69, 126, 34, 38, 55, 19, 27, 72, 33, 66, 32, 127			– 82,004, – 82,005, – 82,006, – 82,007, – 82,009, – 82,010, – 82,011, – 82,012, – 82,013, – 82,014, – 82,015, – 82,016, – 82,017, – 82,018, – 82,019, – 82,020, – 82,021, – 82,025	93.31		

[a]he isolates presented in the table included four reference isolates, indicated by the prefix "ALB-"

[b]In order to provide a better view of information on the Table, since the prefix "MH7" is part of all accession numbers, it was substituted by a dash before each number

[c]Sequences of type material obtained from public databases

[d]Hypothetical protein

2-oxoglutarate dehydrogenase" from the *Bacillus* genus (*Bacillaceae*) (Table 2).

For a further assessment of genetic diversity of the 53 isolates characterized by the *hsp60*-specific amplicons (plus the four reference isolates), a PCR-RAPD analysis was performed (see "Methods"). A total of 87 polymorphic bands (not shown), and an unweighted pair grouping method with arithmetic mean (UPGMA) clustering method based on the similarity profiles of presence/absence of those bands helped generating the dendrograms shown in Fig. 1. The genetic diversity results confirmed intra-genus and inter-genera variation among the isolates, each of which forming a distinct RAPD group. Only two isolates assigned to *Klebsiella* (7 and 12) were 100% identical on their banding patterns (Fig. 1).

Physiological characterization of rhizobacteria

Physiological characteristics of the 63 isolates were investigated by assessing five biochemical phenotypes: cellulase (Cel) chitinase (Chit) and xylanase (Xyl) activities, indolacetic acid (IAA) production, and phosphate solubilization (PhS) (Table 3). Cellulolytic activity was not detected in any of the isolates tested. Considering different combinations of the other four phenotypes, a total of nine functional groups were observed, with each bacterial isolate showing at least one of the activities in vitro (Table 3). Out of all tested isolates, 65.1% (~2/3) presented the functional pattern of absence of hydrolytic enzyme activities and presence of IAA and PhS (Fig. 2 and Table 3), which included members from all the 7 genera of bacteria recovered in this study. Four of the nine functional groups contained three activities simultaneously, comprising a total of 10 isolates: (i) 6 *Serratia* isolates showed Chit, IAA, and PhS, and (ii) one showed Chit replaced by Xyl (i.e., both groups had at least one hydrolytic activity, so that 7 out of the 9 *Serratia* isolates had three activities simultaneously); (iii) one *Pseudomonas* and (iv) two *Enterobacter* isolates showed both enzyme activities, plus the IAA for the former and the PhS for the latter (Fig. 2 and Table 3). When considering the distribution of individual activities among the isolates, the most frequent phenotype was IAA production (88.9% of the isolates), followed by phosphate solubilization (85.7%), chitinolytic (17.4%), and xylanolytic activities (6.3%) (Fig. 2).

Growth promotion assessment

The effects of all isolates on growth promotion variables were assessed by imbibition of cacao seeds in suspensions of each isolate, followed by sowing and cultivation. Significant differences were observed for plant height, shoot, and root dry matters (Table 4 and Fig. 3), whereas no significant differences ($P > 0.05$) were found for the number of leaves (Supplementary Fig. S1). In relation to plant height, no isolate presented any effect of significant increase: 65% did not differ from the control ($P > 0.05$; group A), and a significant decreasing effect was noticed for the remaining 22 isolates (groups B and C). This effect was more pronounced for six isolates from the *Klebsiella* (1), *Enterobacter* (1), *Serratia* (3), and *Bacillus* (1) genera, within a range of 24 to 53% reduction in size (group C; Table 4). Regarding shoot dry matter, approximately half of the isolates showed no significant difference in relation to the control, whereas the other half presented a decreasing effect (groups A and B,

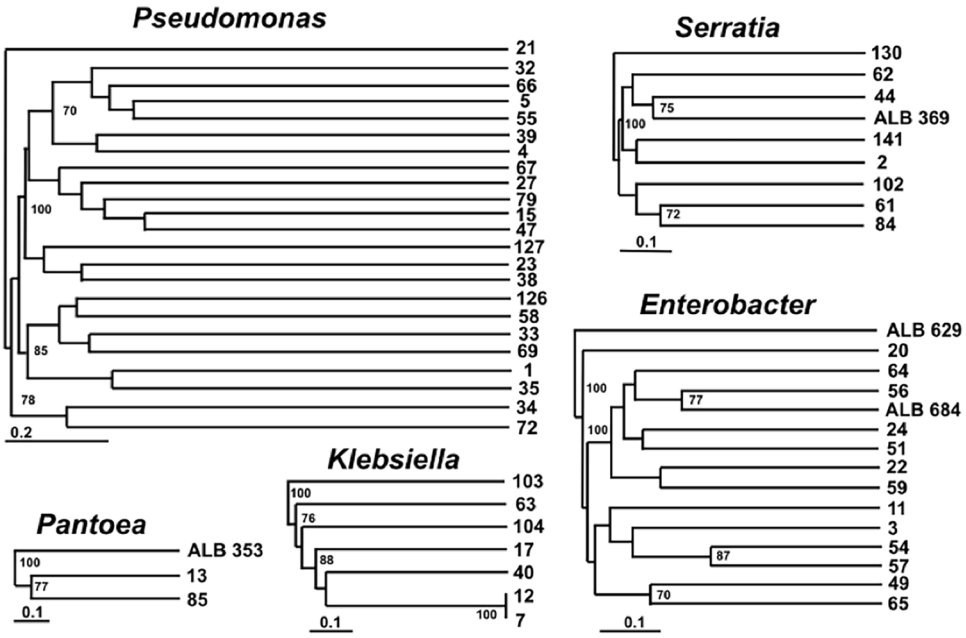

Fig. 1 Diversity analysis of 57 bacterial isolates from cacao based on PCR-RAPD. Dendrograms were constructed using the unweighted pair grouping method with arithmetic mean (UPGMA) and Jaccard's coefficient of similarity. The values presented in the phylogram branches correspond to a bootstrap procedure with 1000 repetitions. The analysis included the 53 rhizobacteria identified by *hsp60*-specific amplicons, plus four reference isolates. The scale below each dendrogram represents the percentage of similarity

Table 3 Patterns of chitinolytic and xylanolytic activities, indoleacetic acid production and inorganic phosphate solubilization in vitro by the culturable isolates from the cacao rhizosphere [a]

Chit	Xyl	IAA	PhS	Isolates	N° isolates	Total
−	−	+	+	*Pseudomonas* (1, 4, 5, 15, 27, 33, 34, 35, 38, 55, 58, 66, 69, 72, 126, 127)	16	41
				Klebsiella (7, 12, 17, 40, 63, 114)	6	
				Enterobacter (11, 20, 24, 49, 54, 56, 57, 59, 64)	9	
				Pantoea (13, 85, ALB-353)	3	
				Bacillus (90, 97)	2	
				Chryseobacterium (110, 124, 133)	3	
				Serratia (130, 141)	2	
+	−	+	+	*Serratia* (2, 44, 61, 84, 102, ALB-369)	6	6
−	−	−	+	*Enterobacter* (3)	1	
				Pseudomonas (39)	1	3
				Bacillus (142)	1	
−	−	+	−	*Pseudomonas* (21, 32, 67, 79)	4	
				Enterobater (22, 65)	2	7
				Klebsiella (103)	1	
+	+	+	−	*Pseudomonas* (23)	1	1
+	−	−	+	*Pseudomonas* (47)	1	1
+	−	−	−	*Enterobacter* (51)	1	1
−	+	+	+	*Serratia* (62)	1	1
+	+	−	+	*Enterobacter* (ALB-629, ALB-684)	2	2

[a]Signals (+) and (−) indicate presence or absence, respectively, of activity for each parameter. "Chit": chitinolytic activity; "Xyl": xylanolytic activity; "IAA": indolacetic acid production; 'PhS': inorganic phosphate solubilization

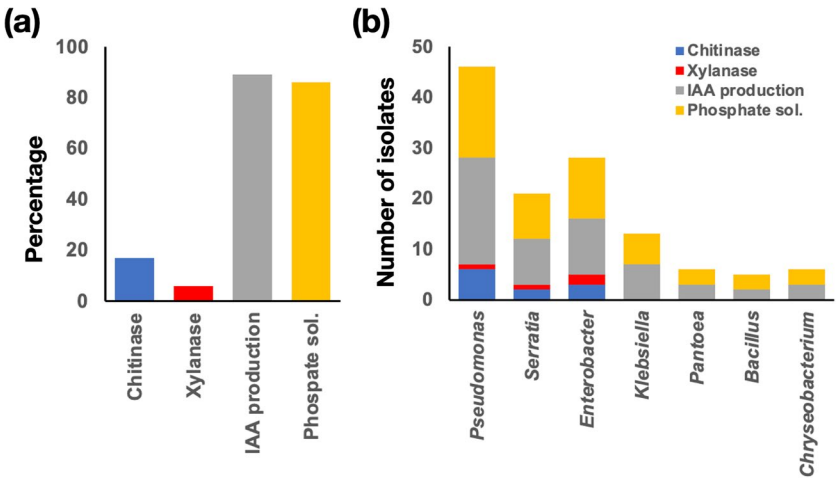

Fig. 2 In vitro activity of culturable isolates from the cacao rhizosphere. (**a**) Percentage; and (**b**) number of isolates with chitinase and xylanase activity, indoleacetic acid (IAA) production and inorganic phosphate solubilization (phosphate sol.) among the culturable rhizobacteria from the cacao rhizosphere. All activities were determined in agar plates containing media with specific substrates for each of the enzymes or traits analyzed. Positive reactions were confirmed with the formation of a halo around the bacterial colony

respectively). Interestingly, 19 of the 22 isolates that had negatively affected plant height were included in the 32 isolates with significantly lower shoot dry matter (Table 4). On the other hand, 18 isolates from the genera *Pseudomonas* (1 + 4 isolates), *Klebsiella* (2), *Enterobacter* (6), *Pantoea* (1), *Serratia* (2), *Bacillus* (1), and *Chryseobacterium* (1) showed significant increases ($P < 0.05$) in root dry matter (Table 4). Specially for isolate "5" (an IAA-producing and P-solubilizing *Pseudomonas*), this beneficial effect was significantly higher than all other isolates, with an increase of 91.8% in root biomass in relation to the control (Table 4). Combining the three growth parameters and the

corresponding statistics performed (i.e., neutral and significantly higher or lower than control), we observed seven groups of statistical significance, in which the isolates were distributed; significantly higher values were observed only for the root dry biomass parameter in three of those groups (Supplementary Table S3). Finally, it was not possible to observe any specific association between taxonomy and the growth promotion parameters, i.e., all genera were present in both significantly and non-significantly different statistical groups generated by the Scott-Knott test for the plant growth variables assessed (Table 4 and Supplementary Table S3).

Despite that our cacao isolates did not provide improvements in the aerial parts of the plants, interestingly, 18 isolates were found to significantly increase root dry mass, but seven of them significantly decreased one or two of the aerial parameters simultaneously (three for plant height, one for shoot dry mass, and three for both); hence, 11 isolates (17.5% of total) displayed root dry biomass significantly higher than the control without decreasing plant height or shoot dry biomass (Table 4 and Supplementary Table S3). In an attempt to further understand this overall pattern, the relationship between root and shoot biomasses was assessed, as this ratio has been revealed interesting aspects of plant–rhizobacteria interactions (Bashan and Dubrovsky 1996; Pérez-de-Luque et al. 2017). We gauged this ratio for the isolates and control treatments, and ranked all treatments based either upon the total plant biomass (i.e., roots + shoot values) or upon roots/shoots (R/S)

Table 4 Growth promotion activity of bacterial isolates from the cacao rhizosphere

Parameters	Groups of isol.[a]	Values[b]	Genera	N° isol.[c]
Plant height (cm)	A (41)	25–30	*Pseudomonas* (1, 4, 5, 15, 21, 23, 27, 32, 33, 35, 38, 39, 47, 55, 58, 66, 67, 69, 72, 79)	20
			Klebsiella (12, 17, 40, 63, 103)	5
			Enterobacter (3, 11, 20, 22, 24, 49, 51, 54, 56, 57, 59)	11
			Pantoea (13)	1
			Serratia (2, 44, 62, 141)	4
			Control	
	B (16)	20–24	*Pseudomonas* (34, 126, 127)	3
			Enterobacter (65, ALB-629, -684)	3
			Pantoea (85, ALB-353)	2
			Serratia (61, 84)	2
			Klebsiella (7)	1
			Bacillus (97, 142)	2
			Chryseobacterium (110, 124, 133)	3
	C (6)	14–19	*Klebsiella* (114)	1
			Enterobacter (64)	1
			Serratia (102, 130, ALB-369),	3
			Bacillus (90)	1
Shoot dry matter ($g.plant^{-1}$)	A (31)	1.0–1.3	*Pseudomonas* (1, 4, 5, 15, 23, 33, 35, 38, 39, 58, 66, 69)	12
			Klebsiella (12, 17, 40)	3
			Enterobacter (3, 11, 20, 22, 24, 49, 51, 54, 56, 59, ALB-684)	11
			Pantoea (13)	1
			Serratia (2, 141)	2
			Bacillus (97)	1
			Chryseobacterium (124)	1
			Control	
	B (32)	0.7–0.9	*Pseudomonas* (21, 27, 32, 34, 47, 55, 67, 72, 79, 126, 127)	11
			Klebsiella (7, 63, 103, 114)	4
			Enterobacter (57, 64, 65, ALB-629)	4
			Pantoea (85, ALB-353)	2
			Serratia (44, 61, 62, 84, 102, 130, ALB-369)	7
			Bacillus (90, 142)	2
			Chryseobacterium (110, 133)	2

Table 4 (continued)

Parameters	Groups of isol.[a]	Values[b]	Genera	N° isol.[c]
Root dry matter (g.plant^{-1})	A (1)	0.4	*Pseudomonas* (5)	1
	B (17)	0.22–0.30	*Pseudomonas* (1, 35, 69, 127)	4
			Klebsiella (17, 103)	2
			Enterobacter (11, 22, 24, 64, ALB-629, -684)	6
			Pantoea (13)	1
			Serratia (2, 141)	2
			Bacillus (97)	1
			Chryseobacterium (124)	1
	C (45)	0.15–0.20	*Pseudomonas* (4, 15, 21, 23, 27, 32, 33, 34, 38, 39, 47, 55, 58, 66, 67, 72, 79, 126)	18
			Klebsiella (7, 12, 40, 63, 114)	5
			Enterobacter (3, 20, 49, 51, 54, 56, 57, 59, 65)	9
			Pantoea (85, ALB-353)	2
			Serratia (44, 61, 62, 84, 102, 130, ALB-369)	7
			Bacillus (90, 142)	2
			Chryseobacterium (110, 133)	2
			Control	

[a]The groups A, B, and C correspond to statistically significant differences ($P < 0.05$) by the Skott-Knott test

[b]The values in this column indicate the range of measures taken for the corresponding parameter (described on left column) that comprise the statistical significance group

[c]The sum of isolates for each genus within a group of statistical significance correspond to the total number of isolates shown between parenthesis in the second column

ratios; applying non-parametric Kruskal–Wallis ranking test in both these variables revealed statistically significant differences only between the extreme positions in the ranks (i.e., 1st and 2nd in relation to the 63rd and 64th), thus indicating the majority of the values in these ranks are not statistically different among them (data not show). Interestingly, though, these results showed that, out of the 64 treatments included in both ranks, the control treatment ranked 9th for total biomass, but 61st for R/S ratio. Therefore, an apparent key effect observed for the rhizobacterial treatments was to increase the root/shoot ratios in relation to the control, although a significant raise in biomass was

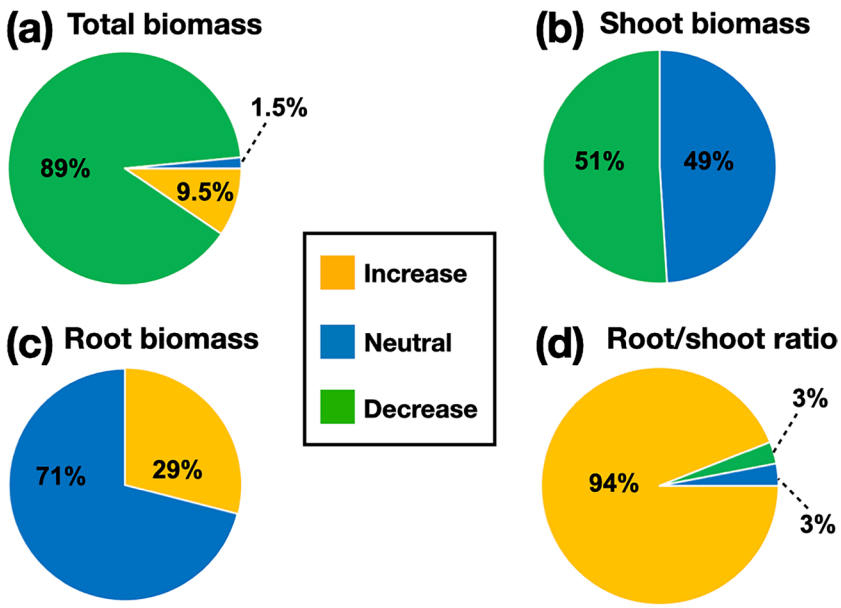

Fig. 3 Effects of rhizobacterial isolates on growth of cacao seedlings in a greenhouse. Percentage of the isolates that affected seedling growth by increasing, decreasing or by having neutral effects on (**a**) total; (**b**) Sshoot; and (**c**) root biomasses; and (**d**) root/shoot ratio. The effects on growth were calculated in comparison with the untreated control. Seeds were inoculated with each bacterial isolate and plants were evaluated 60 days after planting

observed for only a few isolates, based specifically on root assessments (Table 4 and Supplementary Table S3). In fact, the results summarized in Supplementary Table S3 indicated that a relevant portion of the tested isolates (35%) appeared to be deleterious to plant-shoot growth, whereas 65% of the isolates did not show any detectable effect. While half of the isolates (51%) decreased shoot biomass, 71% did not affect root biomass (neutral) and the remaining 29% increased it (Fig. 3), resulting in icreased root/shoot ratios for most isolates (94%), despite a decrease in total biomass by 89% of the isolates (Fig. 3).

Discussion

Severe negative effects on social, economic and environmental settings of the cocoa-producing region of southeastern Bahia (Brazil) were triggered by the witches' broom disease outbreak in 1989 (Perreira et al. 1996), further aggravated by other pathogens. Plant breeding programs coupled with large-scale seedlings production for distribution of improved genotypes to farmers have been the main actions taken to substitute old susceptible plantations with more uniform, productive, and resistant clones (Sodré et al. 2012). In this context, the development of technologies based upon beneficial microbes has shown the potential to aid in improving productivity and stress tolerance of cacao plants (Leite et al. 2013). Likewise, knowledge on growth-promoting rhizobacterial isolates (PGPR) can also add technical advances to the system of mass production of plantlets (Sodré et al. 2012), thus supporting the rehabilitation of cacao plantations. In this work, we studied population densities, genetic and physiological diversity of culturable PGPR associated with the cacao rhizosphere, and their potential to promote growth on seedlings of this crop. The focus of our study on the diversity of a culture-dependent bacterial community is explained by an associated interest in asseessing culturable microbes with potential for further biotechnological developments towards commercial bioproducts useful for cocoa production.

Quantification of culture-dependent bacterial populations and communities revealed generally higher counts in cacao roots for total bacteria and *Pseudomonas*, whereas for *Bacillus*, soil samples showed higher populations, especially in older plantations (Table 1). Since rhizoplane is nutritionally the richest place in the rhizosphere, with ~ 21% of plant photosynthates being released there (Vivanco et al. 2002), those populational differences can be partially explained by the root exudates (Lamb et al. 1996; Elvira-Recuenco and Van Vuurde 2000), whose gradients towards the free/unattached soil help shaping the abundance, richness, and composition of the microbial communities (Trabelsi and Ridha 2013). Bacterial populations in rhizospheres can be 10–1000 times higher than those in adjacent soil (Lugtenberg and Bloemberg 2004), whose fluctuations are not only related to the microenvironment characteristics mentioned above, but also to the genotypes of plants and microorganisms (Campos et al. 2013; Vacheron et al. 2013). The exception to these patterns observed for *Bacillus* revealed different microbial preferences for soil micro-habitats under influence of cacao plants. The data suggest that *Bacillus* spp. tend to display high survival levels in soil, but lower competitive ability on roots. It has been shown that in the rhizosphere of cultivated plants, sporulating bacilli (and Gram-positive cocci) tend to be inhibited, whereas Gram-negative bacteria (such as *Pseudomonas* spp.) tend to be stimulated (Silva and Nahas 2002; Geetanjali and Jain 2016). Despite the fact that *Pseudomonas* spp. can also survive in cultivated/open soils through induction of the known "viable but non-culturable state" (VBNC), a strategy of some Gram-negative bacteria to quickly adapt to environmental stresses and desiccation (Postnikova et al. 2015; Giagnoni et al. 2018), such feature was apparently not enough to overcome the *Bacillus* populations, which tend to produce endospores as survival structures.

Distinct physico-chemical properties and pre-existing levels of micronutrients found in the soil sites (Supplementary Table S1) offer further explanation for the differences we found in bacterial populations. The samples with higher bacterial densities (Old and New CEPLAC) also displayed higher pH, increased contents of Ca, Mg, K, C, Cu, and Mn, lower levels of sand and higher of silt (Supplementary Table S1). Not unexpectedly, soils with high nutrient content tend to exert a positive selection for cells with high growth rates, such as those from α and γ-proteobacteria (e.g., *Pseudomonas* spp., *Enterobacter* spp., *Rhizobium* spp.). Contrariwise, in nutrient-deprived soils and/or those rich in recalcitrant substrates, less quantity and diversity of bacteria are found, in which species with lower growth potential, but higher survival abilities and/or competitive capacity for alternative carbon sources predominate (Smit et al. 2001). This helps explaining not only why *Bacillus* spp. were more abundant in the soil sites of our study, but also why this genus tends to be ubiquitous and cosmopolitan on earth (Loguercio and Argôlo-Filho 2015; Valdivia-Anistro et al. 2016). Size of soil particles has also a great impact on microbial diversity, with greater densities found in silt and clay fractions. Sessitsch et al. (2001) observed that bacteria are usually located in micropores of microaggregates (2–20 mM), which provide favorable growth conditions due to the availability of water, substrate, and gases. Survival and multiplication of bacteria in the rhizosphere interact with physical properties (clay, silt, and sand contents), as well as with organic matter, nitrogen, and calcium carbonate contents (Bach et al. 2010; Flórez-Zapata and Uribe-Vélez 2011).

For a glance on the composition and diversity of the 63 isolates under study, a combined assessment was done based on the *hsp60* gene amplification/sequencing and a PCR-RAPD analysis (Table 2; Fig. 1). The diversity found suggests the experimental design and collection method were sufficient for obtaining an exploitable culturable sample. Such a variability was not unexpected, as studies on phylogenetic relationships and functional traits of rhizobacteria have demonstrated the existence of variation at different taxonomic levels, even for isolates with similar biochemical and niche-occupying patterns (Lennon et al. 2012). Moreover, it is well acknowledged that bacteria represent the richest repertoire of genetic and chemical diversity in nature, especially in tropical ecosystems (Strobel and Daisy 2003; Santos et al. 2019), such as in shaded-cacao plantations (Tchinda et al. 2016). Another interesting evidence of diversity in our set of cacao-related rhizobacteria was the reproducible amplification of non-*hsp60* fragments in some isolates, even using *hsp60*-specific primers (Table 2). Further studies to understand the genomic structures that have conferred higher homology at alternative annealing sites are certainly warranted.

At the depth level of characterization performed, we can consider the cacao isolates as individual operational taxonomic units (OTUs) for the purposes of assessing genetic/phenotypic diversity (e.g., Santos et al. 2019). The retrieval of OTUs belonging to only seven genera (54% belonging to the *Enterobacteriaceae* family) suggests that the use of a single culture medium, even with a generally rich composition, have likely restricted our ability to obtain a greater diversity at this taxonomic level (Lodewyckx et al. 2002). In addition, a host plant selection for specific groups of *Pseudomonas* and *Klebsiella* culturable OTUs might have occurred (Table 2). Despite the natural limitations in accessing the bacterial diversity through culturing approaches, however, the predominance of genera within the *Proteobacteria* phylum, most of which belonging to the gamma (γ) subdivision (*Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, and *Serratia*), has also been observed for various crops, such as *Eucalyptus* sp. (Mafia et al. 2009), loblolly pine (Vonderwell et al. 2001), maize and sugarcane (Santos et al. 2020), tomato, okra, and African spinach (Adesemoye et al. 2008). It is noteworthy that a high frequency of sequence identities below 97% (Table 2) suggests that novel undescribed bacterial species may well be present in this collection of tropical cacao rhizobacteria. Depending on further applications for some of these isolates, an in-depth taxonomic characterization will certainly be required.

The results on the type of taxonomic identification of the rhizospheric bacteria we performed (Table 2) showed that they belong in families bearing PGPRs (Vejan et al. 2016; Duca et al. 2014) that have shown all four physiological activities found for our isolates (Table 3) (Patil et al. 2000; Vazquez et al. 2000; Leontidou et al. 2020; Santos et al. 2020). Rhizobacteria displaying multiple features are well known (Leontidou et al. 2020), so the nine combinatory functional patterns of activities shown in Table 3 were not surprising. However, when considering the growth promotion effects (Table 4), no specific association between certain activities and a beneficial, neutral, or deleterious effect on the cacao seedlings could be identified. Nevertheless, the results interestingly suggested a tendency for cacao plants to have a rhizosphere enriched by culturable bacteria with at least one functional activity usually related to plant development (86 to 89% of isolates), i.e., growth hormone production and/or P solubilization (Vazquez et al. 2000; Gyaneshwar et al. 2002; Khalid et al. 2004; Vacheron et al. 2013). The latter ability is an important feature of PGPRs, since P is the most limiting nutrient for plant growth (Gyaneshwar et al. 2002; Santos et al. 2020). Soils tend to be "phosphorus deficient" because free available P is generally low, even in fertile soils (Richardson 2001). Production of organic and inorganic acids and/or decrease in soil pH allow dissolution of insoluble P (Vassilev and Vassileva 2003). Despite that the IBC and CEPLAC sites were contrasting in P levels (Supplementary Table S1), solubilizing activities were found in isolates from all four sites (Table 3).

Production of growth regulators (such as IAA) has explained the beneficial effect of rhizobacteria in several crops (Vejan et al. 2016; Tsukanova et al. 2017), with these compounds being detected in vitro, in culture media of isolates from *Azospirillum*, *Enterobacter*, *Klebsiella*, *Bacillus*, *Azotobacter*, *Pantoea*, *Pseudomonas*, and *Rhizobium* (Verma et al. 2001; Halda-Alija 2003; Duca et al. 2014). Growth regulators from plant-associated bacteria cause changes in root morphology and influence the absorption of nutrients and water (Pérez-de-Luque et al. 2017). Interestingly, depending on the final IAA concentration (including the plant- and bacterial-derived portions), plant responses may range from beneficial to deleterious effects; when IAA is low, development of roots is stimulated (main, lateral, and hairs), whereas the opposite occurs in high concentrations (Xie et al. 1996; Mafia et al. 2009; Duca et al. 2014; Tsukanova et al. 2017). Nevertheless, some reports have shown that effects of PGPR on growth can be rather independent from auxin levels (e.g., López-Bucio et al. 2007). Our results suggest the need for further studies addressing specific isolates regarding IAA production and their effects on growth of target plants. At this point, it is relevant to highlight that the detrimental effects on plants observed in this study are not related to any phytopathogenic symptom or activity. Further studies specifically addressing potential phytopathogenic effects of the culturable isolates obtained are required, before any technological development procedure based on any of these isolates begins.

The hydrolytic enzyme activities tested were underrepresented in our culturable collection (Table 3). Chitinolytic activity was observed in isolates of *Serratia* and *Enterobacter*, confirming previous reports stating these are main genera of chitinase-producing bacteria, together with *Streptomyces* (Matsuo et al. 1999; Patil et al. 2000). Since chitin is a cell-wall polymer of most phytopathogenic fungi, they are susceptible to the action of chitinase-producing bacteria (Gomes et al. 2000; Majeti and Kumar 2000; León et al. 2009; Santos et al. 2020). Further studies are required to verify the possibility of employing these particular isolates in biological control of relevant cacao pathogens (Hanada et al. 2009), and whether such biocontrol can work as an indirect way of plant growth promotion (Beneduzi et al. 2012; Vacheron et al. 2013; Pérez-de-Luque et al. 2017). Xylanolytic activity was shown by only four isolates (Table 3); xylanase-producing rhizobacteria play a role in mineralization and release of nutrients by degrading complex organic molecules, such as xylan (the main polymer of hemicellulolytic complexes), thereby helping in the decomposition of organic matter (Weselowski et al. 2016). However, these isolates did not promote significant increases in plant biomass of cacao seedlings, suggesting that xylanolytic activity appeared not to be interfering in growth promotion in a detectable manner (Table 4).

Relevant and interesting aspects concerning growth promotion of cacao seedlings by the rhizobacteria under study are worth discussing. First, it was remarkable that none of the response variables related to the aerial part of the plants has shown any significant improvement in relation to the control (Table 4 and Supplementary Table S3). Such phenotypic patterns for rhizobacteria are not uncommon and have been reported in other instances (Bashan and Dubrovsky 1996; Khalid et al. 2004; Leontidou et al. 2020; Pereira et al. 2020). As seen above, the balance between IAA levels produced by the plants and/or the bacteria is what ultimately determines whether there will be growth or inhibition of it. Moreover, optimum physiological levels of auxins that control growth is regulated in a coordinated manner with other plant hormones (e.g., cytokinin, ethylene, abscisic acid), and depend on plant genotype and age (López-Bucio et al. 2007; Mafia et al. 2009; Duca et al. 2014; Park et al. 2015; Tsukanova et al. 2017). Once again, no phytopathogenic effect of any kind that might help explain the observed decrease in growth rates was noticed. Second, the partition of the analysis into roots and shoots data allowed a better glance at the phenomenology of the microbial interaction with cacao plants. Under these conditions, the root/shoot (R/S) dry biomass ratio is an important parameter to address in order to better describe effects of PGPR on the plants as a whole, as this ratio provides an integrated view of a rather complex set of factors and regulatory networks present in PGPRs–plants interactive physiology (Bashan and Dubrovsky 1996; Pérez-de-Luque et al. 2017; Pereira et al. 2020). In this respect, our results revealed an iteresting feature: while 89% of isolates resulted in a total biomass value lower than control (which would point towards a trend in decreasing plant growth rates), 94% of them actually increased the R/S dry biomass ratio (Fig. 3). This confirmed that the root–shoot biomass partitioning is a parameter invariably related to rhizobacterial effects on plants (Bashan and Dubrovsky 1996). Third, and interestingly, such increases in R/S biomass were not promptly and generally visible through direct pairwise comparisons of biomasses between isolates and control, as both neutral or decreasing effects were observed in plant growth (Table 4). Thus, these results suggest that the overall effect of increasing R/S biomass ratio is not necessarily coupled with absolute increases in biomasses. Considering the statistical results obtained for aerial parts- and root-related growth variables assessed (Table 4 and Supplementary Table S3), we claim that measuring both shoots and root biomasses separately (Table 4 and Supplementary Table S3) will be always more effective to account for some quantitatively positive growth promotion effects; for instance, 11 isolates stood out as having significantly improved seedlings biomass, but this could not have been detected if only shoots or total dry matter measurements were taken (Table 4 and Supplementary Table S3).

Fourth, we ought to highlight that our study was performed on non-autoclaved soil, which had likely retained its native microbiota. There is a recognized interaction between inoculated rhizobacteria and the indigenous populations in the soils, which ultimately leads to induction/repression of resident microbial community members, thus altering the synergistic and/or antagonistic effects on the plants (Trabelsi and Ridha 2013; Vacheron et al. 2013). Finally, the simultaneous presence of IAA production and P solubilization was, in fact, abundant among the 63 isolates, being present in all genera identified (Table 3) and distributed among the growth promotion patterns (Table 4). Therefore, neither the presence of IAA- and/or P-related functional phenotypes, nor a preliminary taxonomic identification appear as sufficient indicators (markers) for growth promotion activities, without an understanding of their doses and physiological specificities of the host plants (Vonderwell et al. 2001; Park et al. 2015). Further studies aiming at developing useful associations between phylogenetic signals and microbial functions in ecosystems (Morrissey et al. 2016) are worthwhile to pursue. In addition, these results may motivate new studies to elucidate the impact of those identified shoot growth deleterious rhizobacteria in plants, and to understand whether this detrimental effect may be somehow associated with other plant physiological responses or if they are simply a trade-off with the root growth positive effects that end up increasing the R/S ratios.

In summary, the densities, diversity and activity of the culturable portion of bacteria from the cacao rhizosphere were investigated in this study, aiming at potentially using these isolates to further develop bioproducts that may help improving the initial growth of cacao seedlings, for the rehabilitation of abandoned plantations. Higher densities of *Pseudomonas* were found in the rhizosphere of cacao, whereas *Bacillus* was more numerous in soil. Most genera identified by sequencing a fragment of the *hsp60* gene belonged in the *Pseudomonadaceae* and *Enterobacteriaceae* families. Some of isolates included in the study may represent novel species as the identity of the sequenced fragment was as low as 92% when compared to the most closely related type material deposited in databases. Most isolates in our collection (˜90%) were able to produce IAA and to solubilize phosphate, whereas only a small proportion (˜10%) of them were able to secrete chitinase and xylanase. Despite that most isolates recovered from the cacao rhizosphere appeared to be deleterious to plant height (35%) and total biomass (89%), 94% of all isolates increased the R/S biomass ratio in relation to control, and 29% increased root dry biomass. Further studies should be pursued to gather more information on the true nature of the putative detrimental effects of some cacao rhizobacteria on the plant growth and development, and whether the manipulation of the rhizospheric bacterial community would bring further benefits to cacao, such as (i) plant growth at other stages, (ii) resistance to pathogens, and (ii) tolerance to abiotic stresses.

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Author Contribution ACFS and JTS designed the research and aquired funding. ACMS and JTS conducted the experiments. LLL, JMFLC, PASM, DHR, VC-M and JTS analyzed the data; LLL, DHR, VC-M and JTS wrote the manusript. LLL, JTS, VC-M e JMFL revised and finalized the manuscript. All authors read and approved the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

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