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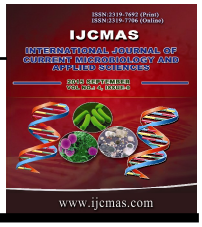


**Original Research Article**

# Phenotypic Diversity of Associative Bacteria Isolated from Roots and Stems of Cacao (*Theobroma cacao*) Tree in Daloa, Côte d'Ivoire

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**A B S T R A C T**

The aim of this work was to investigate the presence of endophytic bacteria inside the roots and stems of young *Theobroma cacao* trees cultivated in Daloa (Côte d'Ivoire) and to characterize the isolates by phenotypic features. 16 young cacao seedlings collected from 8 planting producers and tested *in vitro*, were positive for the bacteria presence. A total of 27 associative bacteria were obtained with 24 from roots and 3from stems. The bacterial density were determined for each fresh root and varied from $4.8.10^3$ to $3.8.10^4$ CFU.$g^{-1}$ of. Based on morphological characteristics, the isolates were found to be Gram positive (93%) and endospore-forming (67 %). We distinguished 4 bacteria groups comprising *Bacillus* (67 %), *Clostridium* (15 %), Actinomycetes (11%) and *Pseudomonas* (7 %) genus. All strains showed a high tolerance to salt stress and resisted well to KCl than NaCl ($p < 0.05$), and grew well at pHs ranged from 3.5 to 10. The mean temperature of growth was 30 °C and 48 to 81 % of strains grew respectively at 4 and 55 °C. All the strains were so sensitive to gentamycin, levofloxacin and doxicyclin even at 10 µg/ml concentration. However, they exhibited a multiple resistance to amoxicillin, azithromycin, sulfamethazol and to various types of heavy metal tested. This research confirms that the *Theobroma cacao* tree is a natural host to diverse associative Rhizobacteria, mostly bacilli, Gram positive and endospore-forming that inhabited inside the roots and stems organs and recognized to promote and protect cacao plants.

**K e y w o r d s**

Isolation, Associative bacteria, *Theobroma cacao*, Phenotypic diversity

## Introduction

*Theobroma cacao* belongs to the family of *Malvacea* and is commonly characterized by three main cultivar groups: Criollo, Forastero and Trinitario. Known to be one of world's most valuable crops, cacao is grown as a sclerophyllous evergreen shrub or tree up to 12 m high and its fruits are about 15-40 cm (Wood and Lass, 1987; Judd *et al.*,

2002). Cultivated worldwide, grown in 58 countries and worth over US$4 billion annually, it is considered to be an important vegetation of environmental, social and economic raisons affecting 25 million peoples in poor rural areas (Van Himme and Snoeck, 2001). The principal producing countries are Côte d'Ivoire (Ivory Coast), Ghana, Nigeria, Indonesia and Brasilia. Currently, Côte d'Ivoire is the leading cacao producer with up to 40 % of world productions.

In Côte d'Ivoire, *Theobroma cacao* was successfully introduced at 19$^{th}$ century in the East regions and expressly distributed in all forest regions (Bardin, 1937; Burle, 1962). Cacao is a well-adapted agro-forestry plantation crops grown in hot and rainy climates. However, it cultivation is mostly threatened by several diseases limiting the productions. Large yield losses due to biological pathogen agent reach of about 100 % in some cacao growing areas in Peru (Evan *et al.*,1998) and 60 % in Côte d'Ivoire (Kebe *et al.*, 2009).

The use of mineral fertilizers or chemical diseases control is considered to be the quickest and surest way of boosting crop production. Meanwhile, their application has many repercussion, as it leads to fruits, ground and water contamination, which is detrimental for human and animal health (Tilak *et al.*, 2005; Jha *et al.*, 2012). Therefore, it's high time to opt for alternative fertilizers or diseases preventions which can be used in agriculture practices without affecting the environment (Jha *et al.*, 2012). Application of Promoting Growth Plant Rhizobacteria (PGPR) as well as associative endophytic bacteria for sustainable agriculture holds immense potential.

The beneficial action of associative endophytic bacteria that infect and colonize space intercellular of plant tissue without causing any apparent damage to the host (De Bary, 1986), consist in producing and delivering growth-promoting substances to plants, stimulating the expression of growth-gens in plant, facilitating the uptake of minerals and water from the soils, limiting the negative influence of toxic heavy metals, exerting an antagonistic action against pathogens and increasing plant resistance to abiotic stresses like drought, flood and salinity (Sturz and Mathson, 1996; Ryan *et al.*, 2008; Dudeja and Giri, 2014; Konate *et al.* 2015). According to several authors, these bacteria are able to make intimate natural association with a wide range woody plant such as *Picea abies* (Shishido *et al.*, 1999.), Citrus plants (Araújo *et al.*, 2002), *Coffea arabica* (Jimenez-Salgado *et al.*, 1997), *Vitis vinifera* (Bell *et al.*, 1995), *Ceratonia siliqua* (Konate, 2007, Konate *et al.*, 2014) and *Theobroma cacao* (Macagnan *et al.*, 2006; Menick *et al.*, 2008 and 2011). Except the work realized by Kebe *et al.* (2009) on indigenous microorganisms of cacao farm soils used as bio-control agent against *Phytophtora*, there is no investigation concerned the finding of endophytic bacteria associated with *Theobroma cacao* tree in West Africa and particularly in Côte d'Ivoire. Thus, we aim (i) to investigate the presence of endophytic bacteria in the vegetative organs of young cacao tree collected in Daloa (Center-West of Côte d'Ivoire) and (ii) to characterize and screen the isolates using phenotypic features.

## Materials and Methods

### Plant material

16 young cacao (*Theobroma cacao*) seedlings cultivated in pouches (Fig. 1) were used to investigate the presence of endophytic associative bacteria in roots and stems. 4 of these joung plants were produced

in the experimental field at the University Jean Lorougnon Guédé in Daloa town in Center-West of Côte d'Ivoire (6, 27° N, -6,53° W), and 12 were collected from 6 farmers of seedling producer in Mimia (about 27 Km of Daloa).

**Isolation of bacterial endophytes from roots and stems**

1g of root from each plant was cut and surface-sterilized successively with Ethanol (70 %) for 5 min, Sodium hypochlorite (25 %) for 5 min and Sodium hydroxyl (4 %) for 10 min (Muzzamal *et al.*, 2012), followed by thorough washing in sterile distilled water and then ground with 1mL of pure water. A volume of 0.1mL of the mixed tissue was directly spotted on YEM medium and other volume was diluted (20x) and used for bacterial enumeration in cacao root (Konate *et al.*, 2014). The stems were successively treated as well as the roots and cut in small piece (0.5 cm) with a sterile blade and placed on sterile YEM agar plates. The cultures were incubated at 30 °C.

**Phenotypic characterization**

All tests were carried out on YEM agar plates. Petri dishes containing defined medium were subdivided into squares and each square was inoculated with 10 µl of YEM broth bacterial culture ($10^8$ cells). After 3 days of incubation at 30 °C, bacterial growth was compared to the controls. Two replicates were done for each treatment.

**Sodium and potassium chloride tolerance**

It was conducted on agar plates at variable concentrations ranging from 1 to 9 % (w/v) of NaCl or KCl concentrations.

**pH tolerance**

Tolerance to pH was tested on YEM plates at different pH values using the buffers HI 7007 (pH 7.01) buffer and HI 70004P **(**pH 4.01) buffer solution for calibration. The medium was buffered with HCl for pH values lower than 7.13 and with NaOH for pH higher than 7.13.

**Temperature tolerance**

YEM agar plates were inoculated as described above and incubated from 4 to 55 °C.

**Antibiotics tolerance**

The intrinsic resistance of strains was determined on solid YEM medium containing the following sterilized antibiotics: Amoxicillin, azithromycin, ciprofloxacin, doxicyclin, penicillin, gentamicin and sulfamethazol at two different concentrations 10 and 50 µg $ml^{-1}$.

**Heavy metal resistance**

The resistance of strains to heavy metals was also determined on solid TY medium. The stock solutions of metals were added at different concentrations to the TY medium and sterilized as follows: $CoCl_2$, 10-50 µg $ml^{-1}$; $AgNO_3$, 10-50 µg $ml^{-1}$; $CuSO_4$, 10-50 µg $ml^{-1}$; $Pb(CH_3COO)_2$, 250-500 µg $ml^{-1}$.

**Catalase test**

It was determined by the method of Graham and Parker (1964).

**Results and Discussion**

**Isolation of root and stem endophytic bacteria**

The isolation of natural endophytic bacteria from the roots and stems of Cacao (*Theobroma cacao*) seedlings harvested after six months of culture was very

successful. 16 young cacao seedlings collected form 8 planting producers and tested *in vitro*, were positive for bacteria presence. We obtained for each provenance a bacterial density arranged from $4.8.10^3$ to $3.8.10^4$ CFU / g of fresh root (Table 1).

We obtained 27 endophytic bacteria with 24 from roots coded BIRC and 3from stems coded BISC. The high number of the isolates (17 %) was originating from Mimia village (Mi 2 and Mi 6) and Daloa-town. The lower number (8 %) was found from UJLoG and Mi 3 samples.

The present work demonstrates clearly that associative bacteria reside inside the internal tissues of the vegetative organs in occurrence roots and stems of cacao without inducing any obvious symptoms. It confirms the results reported by Melnick *et al.* (2011) isolating the bacteria from branch, leaf and pod of *Theobroma cacao*. Endophytes bacteria are ubiquitous and host to most plant species and have been detected in a range of plants woody such as coffee (Jimenez-Salgado *et al.,* 1997), Citrus plants (Araújo *et al.,* 2002) *Vitis vinifera* (Bell *et al.,* 1995) and carob (Konate, 2007, Konate *et al.,* 2014).

**Phenotypic characterization**

The endophytic bacteria selected from the isolation medium were morphologically characterized by Gram reaction (Fig. 2). It was observed that all the isolates were bacilli and mostly Gram positive (93%) and 67 % of them were endospore-forming. Based on morphological traits and respiration activities, we distinguished 4 bacteria groups belonging to *Bacillus* (aerobic and anaerobic), *Clostridium* (anaerobic strict), Actinomycetes (anaerobic strict) and *Pseudomonas* (aerobic strict) genus (Table 2).

Several authors identified *Bacillus sp* and *Pseudomonas sp* as the predominant endophyticous species of different plants (Melnick *et al.,* 2011; Prasad and Dagar, 2014). Many of them demonstrated that endospore-forming bacteria (*Bacillus*), actinomycetes (*Streptomyces* genus), *Clostridium*, *Pseudomonas* and *Azotobacter* sp., colonized the diverse organs and the rhizosphere of cacao tree (Arendsen *et al.,* 1999; Berreto *et al.,* 2008; Melnick *et al.,* 2011; Prasad and Dagar, 2014; Velmurugan *et al.,* 2015; Rahmi *et al.,* 2015).

**Salts tolerance**

Isolates exhibited a wide tolerance to salt stress. They tolerated well KCl than NaCl with a signification degree of $P < 0,05$. 74 and 30 % of strains were unable to grow at the concentration of 9 % respectively of KCl and NaCl (Fig. 3). Strain BIRC3 did not grow in the presence of 2 % NaCl but grew well in YEM medium containing 3 % KCl. Similar osmo-tolerance have been found with the carob endophytic bacteria that were able to grow until 12 % NaCl (Konate *et al.,* 2014). Reva *et al.,* (2002) reported that *Bacillus* sp. isolated from the internal tissues of cotton plants (*Gossipium sp*) presented an optimum growth in the presence of 10 % NaCl.

**pH tolerance**

93 to 100 % of strains grew in lightly acid and neutral pH. 22 % of isolates exhibited and acido-tolerant character since they well in pH 3.5 (Fig. 4). Above pH 7, from 85 to 89 % of strains grew in pH 10. Kulkani and Nautiyal (2000) affirmed that salt tolerance aid symbiotic and endophytic bacteria in tolerance to high pH and temperature. Many actinomyctes grew in the desert soils with pH varied from 3.8 to 11 (Gledhili and Casida, 1969; Singh and Jha, 2015).

**Table.1** Percentage of associative bacteria isolated from roots and stem of cocoa seedlings originating from different localities of Daloa (Center-West of Côte d'Ivoire)

| Accessions | Plants origines | Presence of bacteria (+) or (-) | Bacterial density (CFU.g$^{-1}$ of root) | Type of organe | % of BIRC | % of BISC |
|---|---|---|---|---|---|---|
| 1 | Mi 1 | + | $3{,}58.10^4$ | Roots and Stems | 13 | 33 |
| 2 | Mi 2 | + | $3{,}8.10^4$ | Roots and Stems | 17 | 0 |
| 3 | UJLoG | + | $9{,}4.10^3$ | Roots and Stems | 8 | 0 |
| 4 | Mi 3 | + | $2{,}12.10^4$ | Roots and Stems | 8 | 33 |
| 5 | Mi 4 | + | $3{,}3.10^4$ | Roots and Stems | 8 | 0 |
| 6 | Mi 5 | + | $4{,}9.10^3$ | Roots and Stems | 13 | 34 |
| 7 | Mi 6 | ++ | $2{,}22.10^4$ | Roots and Stems | 17 | 0 |
| 8 | Daloa-town | + | $2{,}5.10^4$ | Roots and Stems | 17 | 0 |
| Total | 16 | 24 from roots and 3 from stems | | | 100 | 100 |

Mi = Mimia village (about 27 Km of Daloa); UJLoG = University Jean Lorougnon Guédé

**Table.2** Analysis of morphological, respiration and catalase activity of the endophytic bacteria associated with cacao tree

| Strains | Respiration | Catalase | Gram | Form | Spore | Species |
|---|---|---|---|---|---|---|
| ***BIRC1*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC2*** | ans | - | + | bacilli | + | *Clostridium sp* |
| ***BIRC3*** | as | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC4*** | as | + | - | bacilli | - | *Pseudomonas sp* |
| ***BIRC5*** | aaf | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC6*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC7*** | aaf | + | + | bacilli | - | *Bacillus sp* |
| ***BIRC8*** | aaf | + | + | bacilli | - | *Bacillus sp* |
| ***BIRC9*** | ans | - | + | bacilli | + | *Clostridium sp* |
| ***BIRC10*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC11*** | aaf | + | + | bacilli | - | *Bacillus sp* |
| ***BIRC12*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC13*** | as | - | + | bacilli | - | *Bacillus sp* |
| ***BIRC14*** | as | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC15*** | ans | - | + | bacilli | - | *Actinomycete sp* |
| ***BIRC16*** | as | + | - | bacilli | - | *Pseudomonas sp* |
| ***BIRC17*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC18*** | ans | + | + | bacilli | - | *Actinomycete sp* |
| ***BIRC19*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BIRC20*** | ans | - | + | bacilli | + | *Bacillus sp* |
| ***BIRC21*** | ans | - | + | bacilli | + | *Clostridium sp* |
| ***BIRC22*** | as | - | + | bacill | - | *Bacillus sp* |
| ***BIRC23*** | aaf | - | + | bacill | - | *Actinomycete sp* |
| ***BISC24*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BISC25*** | ans | - | + | bacilli | + | *Clostridium sp* |
| ***BIRC26*** | ans | + | + | bacilli | + | *Bacillus sp* |
| ***BISC27*** | ans | - | + | bacilli | + | *Bacillus sp* |

as: aerobic strict; ans: anaerobic strict; aaf: aero-anaerobic facultative

**Figure.1** Cacao (*Theobroma cacao*) seedlings cultivated in pouches

**Figure.2** (BIRC20): *Bacillus* sp and (BIRC18): *Actinomyctes*, revealed by Gram reactions

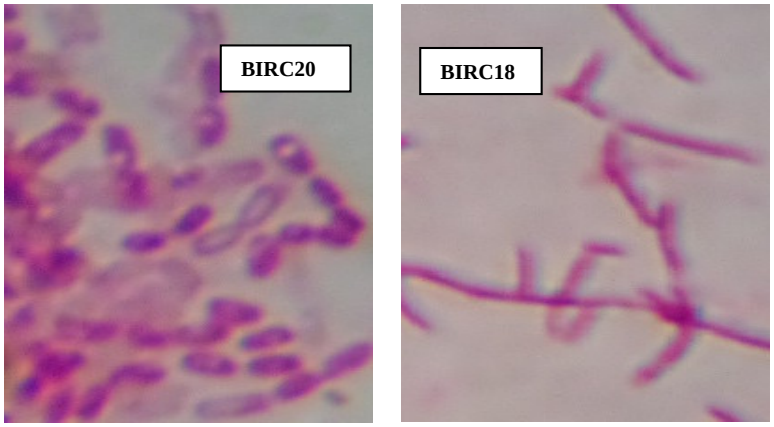



**Figure.3** Tolerance of cacao isolates to different concentrations of NaCl and KCl

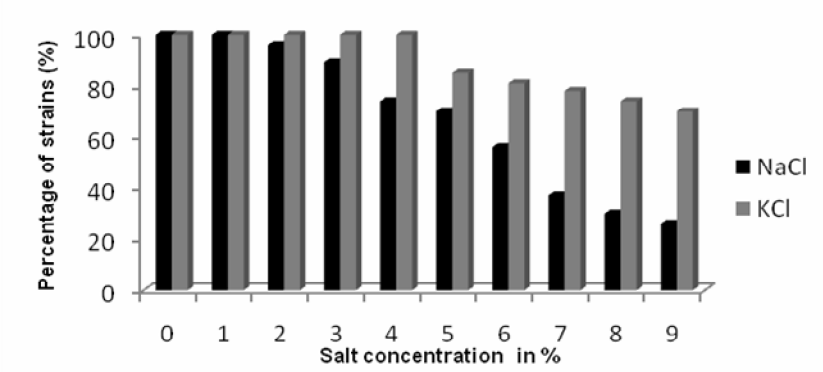


**Figure.4** Influence of pH on the growth of cacao isolates

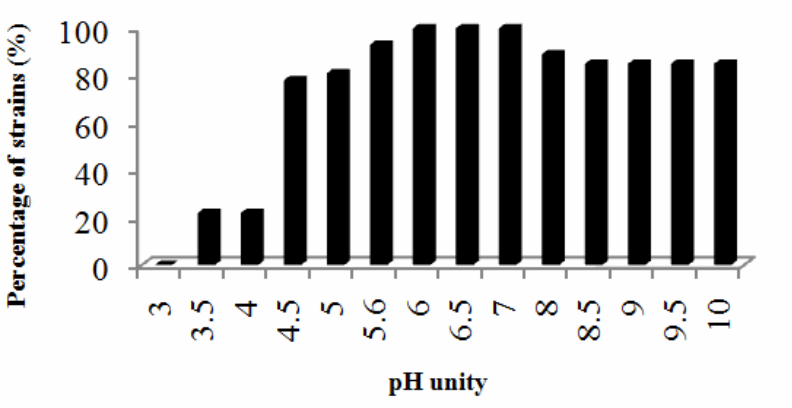



**Figure.5** Effect of temperature on growth of cacao bacteria

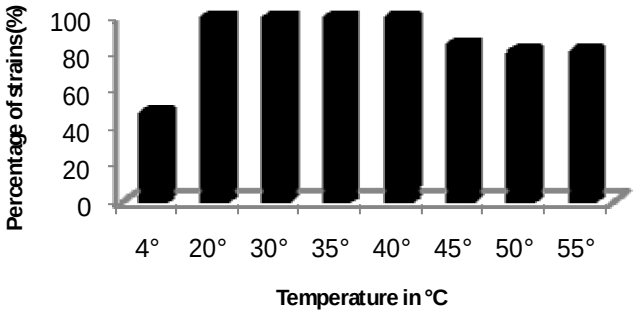



**Figure.6** Effect of diverse antibiotics on growth of cacao strains

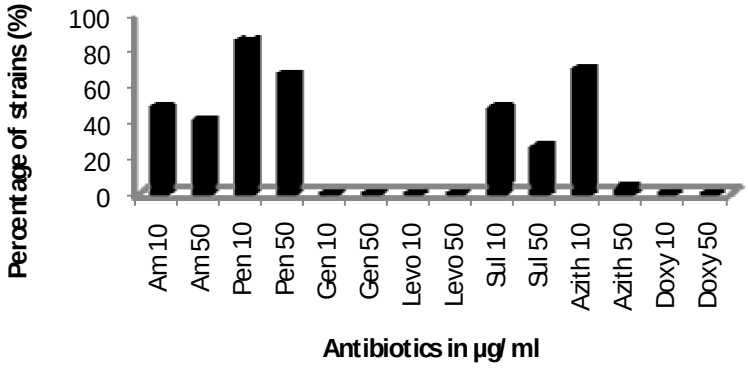
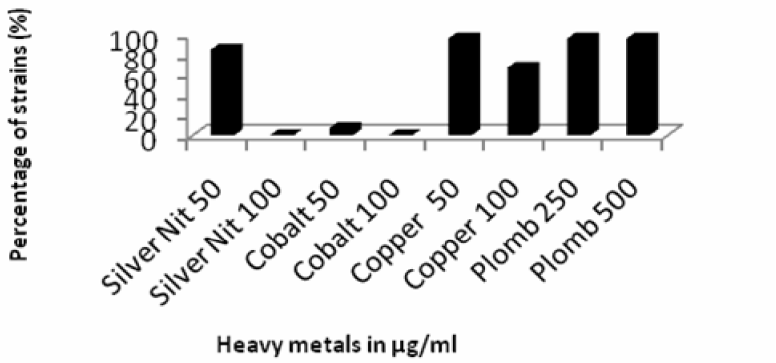


**Figure.7** Effect of various heavy metals on growth of cacao strains




## Tolerance of temperature

The maximum growth of tested strains was obtained from 20 to 40 °C. However, 48 and 81 % of isolates grew well respectively at 4 and 55 °C (Fig. 5). Most of Rhizobacteria as well as rhizobiums native from hot and dry environments of the Sahel savannah and associative bacteria endospore-forming tolerated the high temperature above 60 °C (Egalesham and Ayanaba, 1984; Melnick *et al.*, 2011; Singh and Jha, 2015).

## Antibiotic resistance

None cacao strains grew in the presence of gentamycin, levofloxacin and doxicyclin even at lower concentration (10 µg/ml). However, they exhibited a multiple resistance to various antibiotics tested, amoxicillin, azithromycin, sulfamethazol (Fig. 6). The same result was described for endophytic bacteria isolated from carob tree (Konate *et al.*,2014) and many rhizobacteria belong to genus *Rhizobium*, *Bacillus*, *Pseudomonas*, *Clostridium*, *Burkholderia*, that exhibited a multiple antibiotic resistance (Cole and Elkan,1979; Singh *et al.*, 2013).

## Heavy metal resistance

Figure 6, showed that all isolate were highly sensitive to silver (100 µg/ml) and to Cobalt (50 to 100 µg/ml). However, more than 67 and 85 % of strains exhibited good tolerance to copper (100 µg/ml) and silver (50 µg/ml), 96 % to copper (50 µg/ml) and plomb (250 to 500 µg/ml). Multiple-metal resistance ability of bacteria makes them perfect to apply at metal contaminated sites for bioremediation, as generally, more than one metal is present on such sites (Zhang *et al.*, 2014). Our results were in agreement with lasts rapports on associative and symbiotic bacteria (Mohammed *et al.*, 2000; Konate *et al.*,2014: Zhang *et al.*, 2014).

Traditionally, the mineral fertilization and chemical diseases control have been applied for increasing fruit production in *Theobroma cacao* plantations. However, in recent years, the application of Plant Growth Promoting Rhizobacteria (PGPR) in plant production, protection and adaptation to very poor soils, appear as natural alternative for sustainable agriculture practices. Thus, the Rhizobacteria belong to *Bacillus*, *Pseudomonas*, Actinomycetes, were used for their potential for plant growth promotion and biological control agents of cacao diseases (Macagnan *et al.*, 2006; Kebe *et al.* 2009; Melnick *et al.*, 2011; Prasad and Dagar, 2014).

In conclusion, this research demonstrated

clearly that *Theobroma cacao* tree is natural home to diverse endophytic bacteria that inhabited inside the roots, stems and others organs. The isolates were mostly composed by *Bacillus*, *Pseudomonas* and Actinomycetes that are recognized to increase crops yield and quality and to act as natural antagonist of pathogenic organisms generally in the agro-forestry environment and particularly in the cacao plantations.

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