



Functional and morphological analysis of isolates of phylloplane and rhizoplane endophytic bacteria interacting in different cocoa production systems in the Amazon

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ABSTRACT

Endophytic bacteria colonize different internal tissues of plants without damaging their cells. They can establish themselves in the same niche as other microorganisms and develop antagonistic activities against phytopathogens. There is little research on the functional and morphological characterization of these bacteria in production systems in the Amazon. Thus, the objective of this work was to functionally and morphologically characterize endophytic bacteria isolated from cocoa trees (*Theobroma cacao* L.) and evaluate their antagonistic potential against phytopathogens. A total of 197 endophytic bacteria isolates were obtained from leaves and roots of cocoa plants with different production systems and at different times of the year. The characterization of functional groups consisted of proteolytic, amylolytic and cellulolytic activity and ability to fix nitrogen and solubilize phosphate. Morphological diversity was evaluated mainly according to the following parameters: shape, color, size and elevation of the colony. Thirteen isolates of endophytic bacteria, selected by cluster analysis, were used to evaluate the antagonistic potential in paired trials against four species of phytopathogenic fungi. The largest amount of endophytic bacteria was isolated from the root (95.9%), in the dry season. The most expressive activities with regards to the enzyme index were amylolytic (71.9%), proteolytic (70.2%) and nitrogen fixing (38.6%), respectively. The similarity analysis formed two clusters with isolates CS R 2.4 and CS R 2.25 exhibiting 100% similarity. Five isolates displayed inhibitory activity against phytopathogenic fungi, most notably isolate TS R 2.19, which exhibited antagonistic activity against all fungi and mycelial growth inhibition rates between 25.7% and 50.7%. Understanding the interaction between endophytes in cocoa plants is important as a possible additional tool in biological control. Our studies are incipient and the first to be carried out in different cocoa production systems in the state of Pará, Brazil.

1. Introduction

Plants are commonly associated with several microorganisms. Among these microorganisms, a group of endophytic bacteria may colonize the internal tissues of host plants (Reinhold-Hurek and Hurek, 2011; Ding and Melcher, 2016). Endophytic bacteria can be isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species (Qin et al., 2011). The vast majority penetrate plants through the root (Afzal et al., 2014). However, endophytic bacteria

may enter plants from the phyllosphere through the leaves, via stomata (Senthilkumar et al., 2011). They can be divided into two categories according to where they are found in the host: root endophytic bacteria and leaf endophytic bacteria (Ding and Melcher, 2016).

The same endophytic microorganism can colonize several hosts. However, they may demonstrate functional or ecological specificities in their interactions, being unable to interact with both hosts in the same way (Agrios, 2005). Nevertheless, different species of plants that grow in the same habitat may exhibit a different endophytic diversity,

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Table 1

Characterization of the different cocoa production systems (*Theobroma cacao* L.), in the state of Pará, Brazil, used to collect plant material in this study of endophytic bacteria.

Production System ¹	Characterization	Geographic coordinates
Cabruca systems of cocoa production (CS)	Native forest area with several forest species, several fruit trees including cocoa trees with approximately 35 years of age exploited through the <i>cabruca</i> ² system. Approximately 6.000 cocoa plants with native varieties and CEPLAC ³ hybrids. Area managed with ecological practices. Productivity of approximately 800 g / almond plant.	Latitude: 03°26'41"S; Longitude: 052°48'10"W and Altitude of 254 m
Traditional systems of cocoa production (TS)	Area with previous cultivation of sugar cane (<i>Sacharum officinarum</i> L.), with traditional <i>roça</i> preparation and burning, approximately 6.000 CEPLAC hybrid 17-year-old cocoa plants, susceptible to witch's broom. The area uses chemical control of pests, diseases and invasive plants. Approximate production of 700 g / almond plant.	Latitude: 03°27'54"S; Longitude: 052°56'20"W and Altitude of 144 m
Organic systems of cocoa production (OS)	Area with organic certification seal since 2005. CEPLAC hybrid cocoa trees susceptible to witch's broom, shaded by several forest species. Approximately 4.000 plants aged 18 years. Alternative pest and disease control with natural syrups and organic fertilizer based on bone meal and phosphate rocks. Approximate production of 900 g / almond plant.	Latitude: 03°29'7"S; Longitude: 052°57'45"W and Altitude of 130m
Induced systems of cocoa production (IS)	Area that practices exploitation without fire (<i>roça</i> without burning). Area previously exploited for sugar cane and pasture. Approximately 8.000 CEPLAC hybrid cocoa plants susceptible to witch's broom. Plants aged 18 years and induced via xylem with 5 mL of 0.45 M sucrose six years ago as a form of disease control. This area has been named in this work as induced cocoa and has a yield of 1.100 g / plants.	Latitude: 03°29'24"S; Longitude: 052°57'57"W and Altitude of 180m

¹ CS = Cabruca Systems; TS = Traditional Systems; OS = Organic Systems and IS = Induced Systems.

² Cabruca. Cocoa production system in agroforestry molds.

³ CEPLAC = Executive Committee of the Cocoa Crops Plan.

that is, a different recruitment of endophytes, indicating host specificity (Ding and Melcher, 2016; Ulloa-Muñoz et al., 2020).

Endophytic bacteria play an important role in soil fertility through phosphate solubilization, nitrogen fixation promoting plant growth, biological control and the production of a range of natural products with great potential for use in medicine, agriculture and the industrial sector (Ryan et al., 2008; Etminani and Harighi, 2018). Studies have demonstrated the importance of using these microorganisms in the bioremediation and phytoremediation of contaminated soils (Afzal et al., 2014). Additionally, several beneficial effects have been observed in host plants, such as environmental adaptation to biotic and abiotic stress, adverse nutritional conditions, modulation of plant metabolism, action against herbivory and protection against nematodes and various pathogens, since they may be encountered in the same niche, competing for nutrients and producing antimicrobial substances (Azevedo and Araújo, 2007; Guo et al., 2008; Kaneko et al., 2010; Rohlfis and Churchill, 2011; Lacava and Azevedo, 2014; Miliute et al., 2015).

Several studies have listed isolates of endophytic bacteria from different plant organs in different agricultural crops (Hallman et al., 1997; Ryan et al., 2008; Miliute et al., 2015). Currently, the wide range of existing endophytes makes them a potential tool in the field of agricultural biotechnology (Afzal et al., 2019). This interest relates to the results of several studies in which it was possible to observe the potential of microorganisms that grow in association with plants to promote their growth, due to their antagonism against phytopathogens and pests (Etminani and Harighi, 2018; Young et al., 2013). If these microorganisms were to be applied in agricultural systems, they would represent viable alternatives, based on ecological principles, to replace insecticides and chemical pesticides (Santos and Varalho, 2011).

Few studies on the isolation and characterization of endophytic bacteria have been conducted in cocoa production systems (*Theobroma cacao* L.) in the Brazilian Amazon. Cocoa production has aroused the interest of researchers with regards to the cultivation systems adopted. The cabruca-cocoa agroforestry cultivation system (Lobão and Valeri, 2009), traditional production (Silva Neto and Nakayama, 2013), organic and certified production (Silva et al., 2009) and the farm system, without burning through plant induction, using chemical elicitors (Alves-Júnior and Celestino Filho, 2020; Silva Neto and Nakayama, 2013), are some of the systems used within the production conditions of Medicilândia,

in the state of Pará, which is currently the main producer of cocoa beans in Brazil and also has the highest national productivity (IBGE, 2019).

Studying the endophytic microorganisms that act in these systems and their interactions with cocoa plants is a challenge for agricultural research, mainly due to the importance presented by that culture toward the Brazilian economy and due to the diseases that affect the cocoa tree, especially witch's broom, caused by the *Moniliophthora perniciosa* fungus (Stahel) Aime and Phillips-Mora (2005), a disease that causes great damage to the economy of cocoa-producing countries, as well as the cocoa tree brown rot complex, caused by the *Phytophthora* species (Bastos and Albuquerque, 2013). In addition to these diseases, pests such as the monalunion bug (*Monalunion annulipes* Signoret, 1858) (Hemiptera-Miridae) have caused serious damage by feeding directly on cocoa sprouts and fruits, compromising their production (Sermeño-Chicas et al., 2019).

The most common way of controlling these phytosanitary problems is often the indiscriminate application of chemical pesticides, which generates an imbalance in the environment and soil contamination, which can affect interactions between cocoa plants and endophytic microorganisms present in different production systems. Thus, the objective of this work was to functionally and morphologically characterize endophytic bacteria isolated from cocoa trees in different production systems and to evaluate their antagonistic potential against phytopathogens.

2. Material and methods

2.1. Collection of plant material

Collections were conducted in four cocoa producing areas under different production systems in the state of Pará, Brazilian Amazon, from July 2014 to May 2015 (Table 1). According to the Köppen classification, the predominant climate in the region is "Am" with an annual temperature of 26.3 °C (Dubreuil et al., 2018), annual rainfall around 2.000 mm, relative humidity above 80% and altitude ranging from 134 to 254 m with the predominant soil being Dystrophic *Terra Roxa* soil.

In each production system, samples of leaves and roots were collected from five plants, and their geographical coordinates were obtained (Table 1 and Fig. 1). These collections were carried out at four different times of the year (the period of intersection between rainy and drought seasons; the peak of the drought - in this work called the dry season; the period of intersection between the drought and rainy sea-

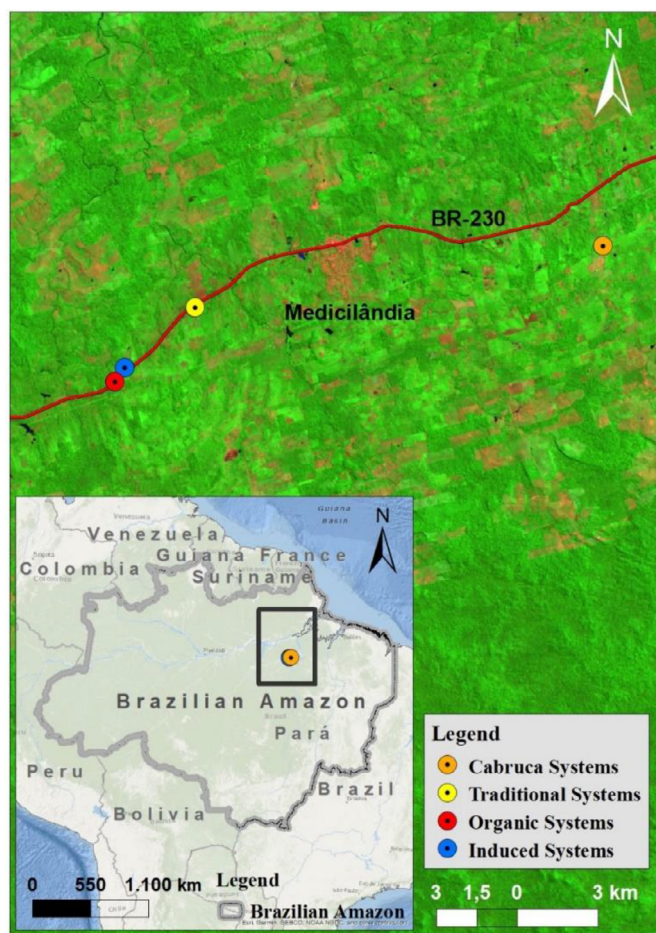


Fig. 1. Location of the four sample collection areas of endophytic bacteria isolates from leaves and roots of cocoa plants (*Theobroma cacao* L.) in Medicilândia, Pará state, Brazilian Amazon.

sons; the peak of the rainy period - in this work called the rainy season). Four healthy leaves from the middle third of each plant, collected obeying cardinal directions, formed a sample composed of leaf per plant. Root fragments collected at four different points of the plant in the layer 0–20 cm deep and 50 cm away from the stem formed a sample composed of roots per plant. All samples were placed in sterile plastic bags and stored in a thermal box containing ice at 4 °C for the conservation of the material that was transported to the laboratory and analyzed within a maximum period of 24 h.

2.2. Isolation of leaf and root endophytic bacteria

The isolation was carried out according to Döbereiner et al. (1995). Leaves were washed in running water with neutral detergent and then dried with absorbent paper. Two fragments of 2 cm in length were removed from each leaf with a box cutter knife. Surface disinfection was carried out through successive immersions in 70% alcohol (v/v) for 1 min, sodium hypochlorite solution 2% (v/v) for 2 min, and three rinses in sterile distilled water for 2 min. The disinfected leaf fragments were macerated in a saline solution (0.85% NaCl), left to rest for 15 min in order to spread the bacteria into the solution (Romeiro, 2001) and 100 µl it was sown in a 523 Kado and Heskett (1970) medium and incubated in a growth oven at 28 ± 1 °C. Triplicates of each material were made.

The procedure for superficial disinfection of root fragments was similar to the one used for leaves. About 1 g of disinfected root was macerated in sterile distilled water (ADE) and left to stand for 15 min,

in order for the bacteria to diffuse into the solution, 1 ml of this macerate was added to 9 ml of saline solution (0.85% of NaCl), and from this dilution (10^{-1}), serial dilutions 10^{-2} and 10^{-3} were performed. Aliquots of 100 µl from each dilution were sown in Tryptic Soy Agar (TSA) (Merck) and incubated in a growth incubator at 28 ± 1 °C. Isolation of fragments of superficially disinfested roots was carried out and they were stirred for 15 min in a saline solution, with three fragments being placed per Petri dish with a 523 medium.

To prove the endophytic nature of the bacteria, the method described by Assis et al. (1998) was used, with some modifications. The leaf or root fragment, after the third rinse of the disinfection process, was divided into two parts. One of them was macerated in a saline solution or ADE as previously described, and the other part was quickly dipped in liquid 523 medium in a test tube and immediately removed. The material in the test tube was incubated in a growth oven under the same conditions as the experiment. In order to prove the absence of epiphytic organisms, the medium could not become cloudy after 48 h of incubation. Another way of proving the endophytic nature was achieved by using 100 µl aliquots of the water of the last rinse of each material sown in a solid 523 medium and incubated at 28 ± 1 °C. Since there was no growth in these plates within 48 h, the isolates obtained were considered endophytic (Naves et al., 2004).

2.3. Preservation of isolates

Colonies characteristic of each morphological type were picked in depletion plates, purified and maintained in microcentrifuge tubes with 30% glycerol at -20 °C, to form a collection of endophytic bacteria from the cocoa tree in the UFPA Agricultural and Forestry Phytopathology Laboratory. Preservation of the isolates using the Castellani method (1967) was also carried out, using tubes with screw caps and sterile distilled water; and in an inclined tube containing NYDA medium (dextrose 10 g, meat extract 3 g, yeast extract 5 g, agar 18 g / l of distilled water) preserved at 4 °C.

2.4. Functional characterization of isolates

The isolated and purified bacterial strains were analyzed for enzymatic production, biological fixation of atmospheric nitrogen and the ability to degrade phosphate minerals, according to the methodology of analysis of functional groups of microorganisms, described by Albino and Andrade (2006). Cross-sectional measurements of halos and colonies were taken to calculate the enzyme index.

The amilolytic activity of the isolates was assessed via the same method, using the AMILO culture medium (Pontecorvo et al., 1953; Oliveira et al., 2010) and development was carried out by applying 10% lugol solution to the plates. The lugol reacts with the starch, producing a purple color and allowing for the visualization of the degradation halos. The production of cellulases followed the same methodology, applying the isolates in the CELULO culture medium (Wood, 1980). Development is achieved by applying 0.5% Congo red solution to the plates, removed with saline solution (0.85% NaCl) after 30 min. Cellulose becomes red and the degradation halos appear yellow.

Phosphate solubilizers were analyzed by inoculating the isolates onto the SF culture medium (Sylvesterbradley et al., 1982), which does not require any further development, but requires an eight-day incubation period. Therefore, Petri dishes should receive a greater volume of culture medium. The medium, which is opaque at the moment in which it receives the bacteria, becomes clear around colonies capable of solubilizing phosphate crystals. The phosphate solubilization index (ISF) was calculated through $ISF = A/B$, where A was the diameter of the halo (phosphate solubilization) including the colony and B consisted only of the diameter of the colony (Montañez et al., 2012).

The biological fixation of N was analyzed by inoculating the isolates in the BNF culture medium (Döbereiner and Day, 1976). Since the N-fixing microorganisms are microaerophilic, the plates must be wrapped

in plastic film and incubated at 28 ± 1 °C for five days. N fixation is revealed by the appearance of a blue halo around the colonies.

2.5. Morphological characterization

The endophytic bacteria kept in glycerol under refrigeration were picked by the streak method in Petri dishes containing the NYDA medium and kept in a growth oven at 28 ± 1 °C, in order to perform the morphological characterization according to the methodology of [Hungary and Silva \(2011\)](#), using the following parameters: growth manifestation, colony shape, colony elevation, border type, colony surface, mucus production, consistency, colony color, as well as optical detail and the catalase test. The morphological characterization experiment was carried out in duplicate. The Gram stain test was performed according to the method described by [Tortora et al. \(2017\)](#).

2.6. Clusters analysis for grouping of endophytic bacteria

With the information on the functional, biochemical and morphological characterizations of each isolate of endophytic bacteria, 57 isolates that performed at least two functional activities were chosen for the analysis of clusters. All information was transformed into a binary spreadsheet and subsequently used to obtain a similarity dendrogram, calculated using the Jaccard coefficient and grouped using the UPGMA (Unweighted Pair-Group Method with Arithmetical Average) algorithm. For these analyses, the e-Past software for Ecology was used ([Hammer et al., 2001](#)).

2.7. Selection of endophytic bacteria with antagonistic action to phytopathogens

Thirteen isolates of endophytic bacteria chosen through cluster analysis and which presented three or more functional activities were used to select strains of interest with antagonistic potential. The antagonism of endophytic bacteria was evaluated according to the methodology adopted by [Shiomi et al. \(2008\)](#) against four species of phytopathogenic fungi: *Fusarium* sp., *Cercospora coffeicola*, *Rhizoctonia solani* and *Pestalotiopsis guepinii*, all belonging to the mycology of the Agricultural and Forestry Phytopathology Laboratory at UFPA.

A phytopathogen disk of 0.5 cm in diameter was inoculated in the center of the Petri dish, containing a PDA culture medium (Potato, Dextrose, Agar). Subsequently, two streaks of the endophytic bacteria were made on each side of the plate, at a distance of two and a half centimeters from the phytopathogen, the total diameter of the plate being 9 cm. The control treatment consisted of placing, on the Petri dish, only one disk of medium culture containing the phytopathogen. For the evaluation of the inhibition zone, the established criteria was the period of time necessary for the mycelium of the pathogen, without the presence of the endophytic bacteria, to develop over the entire medium culture with a limit of 12 days. The tests were performed in triplicate.

The inhibition index of the endophytic bacteria was assessed by measuring the area of the phytopathogen in comparison with the control area, and the average radius of the colony and the Relative Inhibition were calculated, using the formula: $I.R. (\%) = (RC - RX) \times 100$, where:

IR = Relative Inhibition in percentage;

RC = Colony radius of the pathogen in the control treatment.

RX = Colony radius of the pathogen paired with the isolate of endophytic bacteria, according to the methodology adopted by [Shiomi et al. \(2008\)](#).

2.8. Statistical analysis

For the functional analysis, the experiment was completely randomized with three repetitions, with each repetition being constituted by a Petri dish. The data obtained was transformed (\sqrt{X}) and subjected

to analysis of variance, and the significant means were discriminated by the Tukey test ($P > 0,05$) and compared to each other using Microsoft Excel and Assisat 7.7 software Beta ([2016](#)). For the morphological characterization, a completely randomized design with 3 replications was adopted. For cluster analysis, a similarity dendrogram was calculated using the Jaccard coefficient and grouped using the UPGMA algorithm, using the e-Past software for Ecology ([Hammer et al., 2001](#)). For the antagonism test, a completely randomized design with 3 replications was used and the data were submitted for analysis of variance and Scott-Knott test at a level of 5% probability.

3. Results and discussion

3.1. Isolates prospected at different times and cocoa production systems

Endophytic bacteria colonize the interior of plants and interact with their hosts in an intimate and beneficial relationship. The functional and morphological characterization of endophytic bacteria isolates contributes toward taxonomy and helps to understand the functions of these microorganisms in their ecological niche. Additionally, these bacteria can activate the plant's endogenous defense mechanisms and perform biocontrol activities in nature ([Etminani and Harighi, 2018](#); [Hallmann et al., 1997](#)). In this work, a total of 197 endophytic bacteria were obtained from the internal tissue of leaves and roots of cocoa plants in four different production systems in the state of Pará, Brazilian Amazon.

The isolates were collected from different tissues of the plant, with 95.9% of isolates coming from the root and 4.1% of isolates coming from the leaf. There were no significant effects ($P > 0.05$) when comparing the total number of root isolates in the four different cocoa production systems. However, the number of isolates obtained from the root differed significantly ($P > 0.05$) in relation to those obtained from leaves ([Fig. 2](#)).

Some studies have shown that endophytic bacteria are able to penetrate the plant from the phyllosphere via leaf stoma ([Ali et al., 2012](#); [Compant et al., 2010](#)). However, the main organ from where endophytic bacteria penetrate their host is the root ([Afzal et al., 2014](#)). The data in the present study corroborates these studies, with a significant amount of endophytic bacteria having been isolated from the root. This distribution of the endophytic community concentrated in the roots; follows the same colonization pattern of endophytic bacteria isolated from sugar cane ([Oliveira et al., 2009](#); [Silva et al., 2012](#)) and rice ([Prakamhang et al., 2009](#); [Cardoso et al., 2010](#)). This is due to the fact that, in the region of root growth, there is a greater concentration of compounds exuded by plant roots, loaded with energy sources and nutrients necessary for bacterial cell cycles ([Silva et al., 2012](#)). The trend of preferential colonization of roots reflects the presence of high levels of nutrients in the rhizosphere and rhizoplane, which can be used for bacterial growth and metabolic activity ([Oliveira et al., 2009](#)).

Studies carried out in Malaysia with cocoa plants obtained 103 endophytic bacteria isolates from different parts: 36.9%, 34.9% and 28.2% isolated from leaves, branches and fruits respectively ([Alsultan et al., 2019](#)). [Ouattara et al. \(2019\)](#) isolated a total of 218 endophytic bacteria isolates from roots, stems and leaves in cocoa seedlings. Corroborating our results, [Melnick et al. \(2011\)](#) isolated endophytic bacteria that form endospores from the leaves, fruits, stems and floral cushions of cocoa plants. Therefore, it is known that endophytic bacteria can be isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species ([Qin et al., 2011](#); [Compant et al., 2011](#)).

Regarding the production system, the induced system and the organic system presented, respectively, a slightly greater amount of isolates prospected in roots. In addition to the aforementioned facts with regard to the greater amount of root isolates, the induced system in this work is composed of plants with an application of 0.45 M sucrose via xylem, which activates endogenous defense mechanisms of plants and can facilitate interaction as with endophytic microorganisms ([Vieira and Valle, 2012](#)).

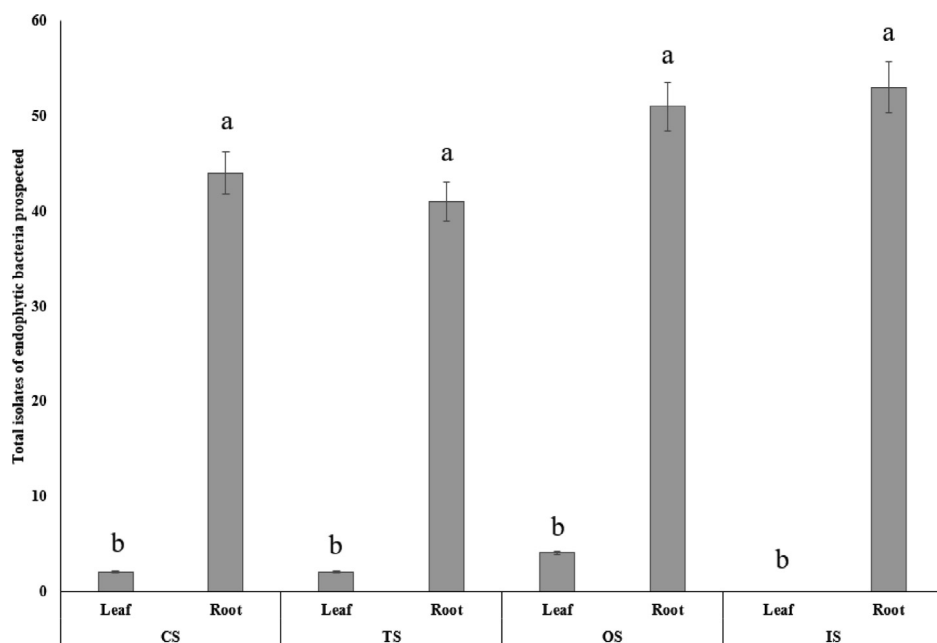


Fig. 2. Number of endophytic bacteria isolates obtained from leaf and root in four collections from July 2014 to May 2015. CS = Cabruca Systems; TS = Traditional Systems; OS = Organic Systems and IS = Induced Systems.

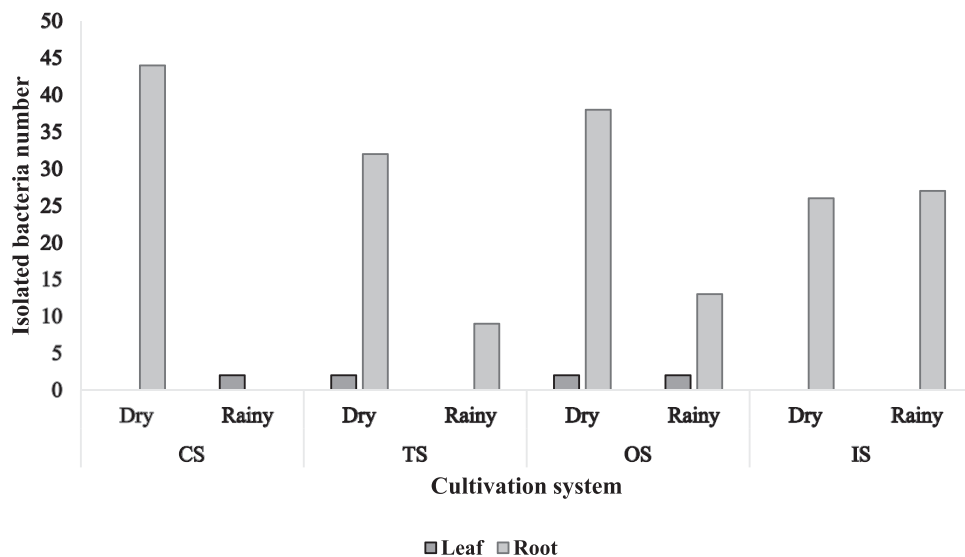


Fig. 3. Number of endophytic bacteria isolates obtained from leaf and root in four different cocoa cultivation systems and over four collections in the periods from July 2014 to May 2015. CS = Cabruca Systems; TS = Traditional Systems; OS = Organic Systems; IS = Induced Systems. Dry season, less water availability; rainy season, greater availability of water.

The collections were carried out at different times of the year, encompassing the dry and rainy seasons. In this context, the dry season presented the largest amount of prospecting endophytic bacteria isolates with 144 (73.1%), against 53 (26.9%) in the rainy season. These results demonstrate a trend highlighting the importance of the dry season for the activity of endophytic bacteria in the cocoa crop under Amazonian conditions (Fig. 3). Endophytic bacteria that inhabit the phylloplane tend to suffer more interference, with increased precipitation compared to those that inhabit the rhizosphere. However, it is known that in the Amazonian winter, the rainfall regime is vigorous, with a precipitation volume of approximately 1800 mm, concentrated between the months of February and May. This precipitation can affect phylloplane endophytic bacteria, due to constant leaf wetness. On the other hand, in the dry season, the rhizosphere does not undergo sudden changes in temperature and humidity, since the cocoa production systems in the region are mostly shaded by forest species, or are in agroforestry systems. Soil bacterial communities are known to be more resistant to climate change than leaf bacterial communities (Ren et al., 2015).

When analyzing the average of isolates per collection period, taking into account four collections spaced in time between dry and rainy season, different ecological characteristics are observed between the production systems. Organic Systems (OS) were the production systems in which the best distribution of isolates occurred, presenting an ecological balance with characterized samples of roots and leaves in all evaluated periods (Fig. 3). Organic production systems tend to exhibit a greater ecological balance, since the control of pests and diseases occurs in an alternative way and fertilization comes from organic sources. The benefits of organic cocoa production systems, which do not use chemical pesticides, have been demonstrated in the region of the study (Alves-Júnior and Celestino Filho, 2020). Without the use of chemicals, communities of endophytic and epiphytic microorganisms are more easily established.

The IS presented results similar to those of the OS, but there were no isolates of leaf endophytic bacteria. This system was the most expressive in terms of root isolates (Fig. 3). This fact may be related to the activation of the plants' defense mechanisms, since this area is induced

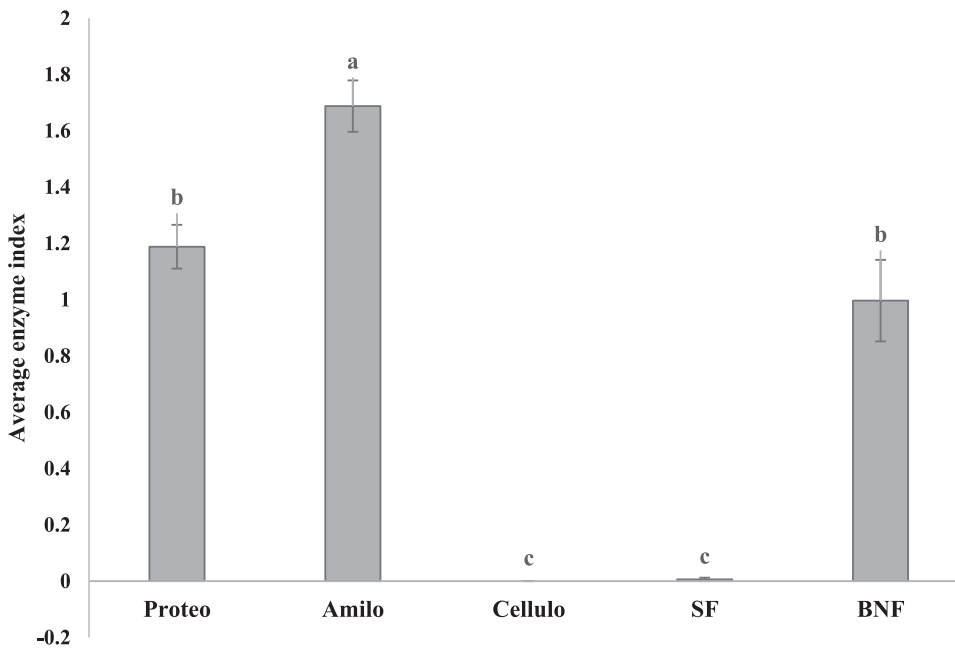


Fig. 4. Average Enzyme Index (IE) of the endophytic bacteria isolates obtained from the internal tissue of the leaf and root of the cocoa tree. Proteo = Proteolytics; Amilo = Amylolytics; Cellulo = Cellulolytics; SF = Phosphate Solubilizers and BNF = Biological Nitrogen Fixators.

with chemical elicitors (e.g. 0.45 M sucrose), and studies show that this inducer works as an activator of endogenous resistance mechanisms in cocoa trees (Vieira and Valle, 2012; Vieira et al., 2013). Interactions between endophytic bacteria and their hosts have been reported (Rosenblueth and Romero-Martínez, 2006). Understanding the mechanisms and functions of endophytes can contribute to agricultural management practices with regard to the biocontrol of plant diseases, bioremediation, plant nutrition and promotion of plant growth (Compant et al., 2005; Sessitsch et al., 2012).

3.2. Functional and morphological cluster analysis

Selective culture mediums are widely used to understand the role of microorganisms in their ecological niche. A total of 197 isolates of endophytic bacteria, isolated from cocoa trees in different production systems, were subjected to an analysis of functional groups to determine the functions that these organisms play in the plant.

The most expressive functional group was that of amylolytic bacteria, with an average enzyme index of 1.7. This group differed significantly ($P > 0.05$) in relation to the functional group of proteolytic bacteria, which obtained an average of 1.2, and nitrogen fixers, with an average of 1.0. Proteolytic bacteria and nitrogen fixers did not differ significantly from each other (Fig. 4 and Table 2). Phosphate solubilizers displayed a negligible number of isolates and microorganisms with cellulolytic activity were not described in this work.

Among the isolates chosen for the morphological characterization, 71.9% displayed amylase activity. Bacteria are producers of the amylase enzyme, which degrades starch until it becomes glucose, which is used as an energy source (Oliveira et al., 2007). Glucose is involved in the plant's endogenous defense routes (Vieira and Valle, 2012).

Another source of nitrogen in ecosystems lies in the decomposition of proteins. In the soil, proteolytic microorganisms are found in small numbers, compared to those that are part of the other functional groups. They perform important functions in the ecosystem, playing a considerable part in protein decomposition (Sanomiya and Nahas, 2003). The functional group of proteolytic bacteria that obtained an IE average of 1.2, with 70.2% of isolates developing this activity, was also the only group that showed a significant difference in relation to the number of isolates collected and periods of collection.

The third collection produced the largest number of endophytic bacteria with proteolytic activities. Regarding the Enzyme Index, there was no observed difference between the bacteria that showed nitrogen fixing activity with IE 1.0 and the proteolytic ones. However, only 38.6% of the endophytic bacteria isolated in this study exhibited this activity (Fig. 4 and Table 2).

A total of 197 endophytic bacteria isolates were morphologically characterized. However, Table 2 shows the results of 57 isolates that were chosen for performing at least two functional and/or biochemical activities.

The Gram stain test showed 63.2% of Gram-positive endophytic bacteria with a majority of rod-shaped isolates. These results corroborate the findings of Alves et al. (2014), but differ from those found by Lima (2013), who, working with the morphophysiological and genetic characterization of endophytic bacteria isolated from corn, verified 75% of the isolates as Gram-negative. Gram-positive endophytic bacteria, mainly species of the genus *Bacillus*, have been reported to be the most commonly found (Bacon and Hinton, 2007). On the other hand, Gram-negative endophytic bacteria can act in biological control (Kobayashi and Palumbo, 2000).

The morphological characterization of endophytic bacteria is important as a first approach in the evaluation of the diversity of bacterial populations. Our work carried out a morphological characterization of 197 isolates, according to the methodology of Hungary and Silva (2011), using several parameters to allow for the morphological grouping of similar individuals.

The main shape of the bacterial colonies was circular, with 57.9%. As for color, the cream color of bacteria isolates predominant, with 80.7%. The predominant size of the colonies was moderate, with 45.6%, whereas the predominant elevation was flat, with 61.4%. The morphological characterization showed variation among the isolates of endophytic bacteria, which was an expected hypothesis in itself, when dealing with isolates prospected in different habitats.

The Jaccard similarity coefficient showed two distinct groups (clusters) with high variability between isolates of endophytic bacteria. We highlight the clusters between isolates CS R 2.4 and CS R 2.25, both obtained from the Cabruca System, from the root in the second collection, which displayed 100% similarity (Fig. 5).

The CS R 2.4 and CS R 2.25 isolates exhibited the second highest degree of similarity, with the CS R 2.9 isolate exhibiting approximately

Table 2

Functional activities, Gram reaction, catalase production and morphological characteristics of endophytic bacteria isolated from cocoa plants.

Isolate Codes	Functional Activity					Gram Reaction	Catalase Production	Colony Characteristics			
	Prot.	Am.	Cel.	BNF	SF			Form	Color	Size	Elevation
OS L 2	+	+	-	-	-	+ ve	+	Circular	Cream	Abundant	Flat
CS L 3	-	++	-	-	-	+ ve	+	Circular	Orange	Small	Convex
OS L 4	++	+	-	-	-	+ ve	+	Circular	Cream	Small	Flat
OS R 1.2	-	++	-	-	-	+ ve	+	Pointed	Cream	Small	Flat
OS R 1.3	-	+	-	-	-	- ve	+	Circular	Cream	Abundant	Lens
IS R 1	+	-	-	-	-	- ve	+	Pointed	Cream	Moderate	Flat
OS R 2	+	-	-	-	-	- ve	-	Circular	Cream	Abundant	Flat
OS R 2.1	+	-	-	-	-	- ve	-	Circular	Cream	Moderate	Lens
CS R 2.1	+	++	-	-	-	+ ve	+	Circular	Cream	Moderate	Flat
TS R 2	-	+	-	-	-	+ ve	+	Irregular	Cream	Abundant	Lens
TS R 2.2	++	-	-	++	-	+ ve	+	Pointed	Cream	Moderate	Flat
TS R 2.3	-	-	-	++	-	+ ve	+	Circular	Cream	Abundant	Convex
TS R 2.4	+	+	-	-	-	+ ve	+	Irregular	Cream	Abundant	Lens
IS R 2.1	-	+	-	-	-	- ve	-	Irregular	Cream	Abundant	Raised
OS R 3.1	-	-	-	++	-	+ ve	-	Circular	Cream	Moderate	Lens
IS R 3	+	++	-	-	-	- ve	+	Circular	Pink	Moderate	Convex
IS R 3.1	+	-	-	+	-	- ve	-	Circular	Cream	Moderate	Lens
OS R 4	+	+	-	-	-	- ve	+	Circular	White	Scarce	Lens
IS R 4.1	-	+	-	-	-	+ ve	+	Circular	Cream	Small	Flat
IS R 4.2	+	+	-	-	-	+ ve	+	Circular	White	Moderate	Flat
OS R 1.4	-	+	-	-	-	+ ve	+	Irregular	White	Abundant	Flat
OS R 1.7	+	+	-	-	-	+ ve	+	Circular	Cream	Small	Lens
OS R 1.8	+	+	-	-	-	+ ve	+	Pointed	Cream	Moderate	Flat
CS R 1.4	+	-	-	-	-	+ ve	-	Circular	Cream	Moderate	Lens
CS R 1.6	+	+	-	-	-	- ve	+	Circular	Cream	Small	Flat
OS R 1.21	-	-	-	+	-	- ve	+	Circular	White	Abundant	Lens
OS R 1.22	-	-	-	+	-	- ve	-	Irregular	Cream	Abundant	Lens
OS R 1.27	++	-	-	-	-	+ ve	+	Irregular	Cream	Abundant	Flat
TS R 1.4	-	+	-	-	-	- ve	-	Irregular	Cream	Moderate	Flat
IS R 2.8	++	+	-	-	-	- ve	+	Irregular	Cream	Abundant	Flat
CS R 2.4	+	+	-	-	-	+ ve	+	Circular	Cream	Moderate	Flat
CS R 2.6	+	+	-	++	-	+ ve	+	Circular	Cream	Moderate	Flat
CS R 2.9	+	+	-	++	-	+ ve	+	Circular	Cream	Moderate	Flat
CS R 2.12	+	+	-	+	-	+ ve	+	Circular	White	Abundant	Flat
CS R 2.13	+	+	-	+	-	- ve	+	Irregular	Cream	Small	Flat
CS R 2.14	+	++	-	++	-	+ ve	+	Circular	Cream	Moderate	Lens
CS R 2.17	+	-	-	+	-	+ ve	+	Irregular	Cream	Small	Flat
CS R 2.21	+	+	-	-	-	+ ve	+	Circular	Cream	Moderate	Lens
CS R 2.24	+	-	-	++	-	+ ve	-	Pointed	Cream	Small	Flat
CS R 2.25	+	+	-	-	-	+ ve	+	Circular	Cream	Moderate	Flat
TS R 2.8	+	+	-	+	-	+ ve	+	Irregular	Cream	Moderate	Flat
TS R 2.9	+	+	-	-	-	+ ve	+	Circular	Cream	Abundant	Flat
TS R 2.13	+	+	-	-	-	+ ve	+	Pointed	Cream	Small	Flat
TS R 2.18	+	++	-	++	-	- ve	-	Circular	Cream	Moderate	Flat
TS R 2.19	+	-	-	++	-	+ ve	+	Irregular	White	Small	Flat
TS R 2.27	+	+	-	-	-	+ ve	+	Irregular	Cream	Abundant	Convex
OS R 3.6	+	+	-	-	-	+ ve	+	Irregular	Cream	Abundant	Raised
TS R 3.2	-	+	-	+	-	- ve	+	Pointed	Cream	Small	Flat
IS R 3.2	-	-	-	++	-	- ve	-	Pointed	Yellow	Moderate	Flat
IS R 3.5	+	+	-	-	-	+ ve	+	Circular	White	Moderate	Flat
IS R 3.9	-	+	-	-	-	+ ve	+	Circular	Cream	Moderate	Flat
IS R 3.11	++	+	-	-	-	+ ve	+	Circular	Cream	Moderate	Lens
OS R 4.4	+	+	-	++	-	- ve	-	Circular	White	Moderate	Flat
OS R 4.5	+	-	-	++	-	- ve	+	Circular	Cream	Moderate	Lens
IS R 4.5	+	+	-	-	-	+ ve	+	Pointed	Cream	Moderate	Flat
IS R 4.6	-	+	-	++	-	- ve	+	Circular	Cream	Small	Lens
IS R 4.7	-	+	-	++	-	- ve	+	Pointed	Cream	Abundant	Flat

Prot. = Proteolytic; Am. = Amylolytic; Cel. = Cellulolytic; BNF = Biological Nitrogen Fixators; SF = Phosphate Solubilizer. Positive reaction (+); Strong positive reaction (++) and Negative reaction (-); + ve = Gram positive and - ve = Gram negative. CS L = Cabruca Systems Leaf; CS R = Cabruca Systems Root; TS R = Traditional Systems Root; OS L = Organic Systems Leaf; OS R = Organic Systems Root; IS R = Induced Systems Root.

93% similarity, and the third highest degree came from isolate IS R 3.9, with 90% similarity, the last one being from the Induced System from the third collection. All isolates were obtained from the root. Our results differ from those found by [Lira-Cadete et al. \(2012\)](#) and [Naik et al. \(2008\)](#), who found peak degrees of similarity of 83% and 80%, respectively. However, these authors worked with phosphate solubilizing bacteria.

3.3. Antagonistic activity of endophytic bacteria isolates from cocoa plants

One of our hypotheses was that isolates of cocoa tree endophytic bacteria could promote antagonistic activities in relation to phytopathogens, as they are in the same niche, competing for nutrients and producing antimicrobial substances involved in biological control. This hypothesis has already been tested in several studies, such

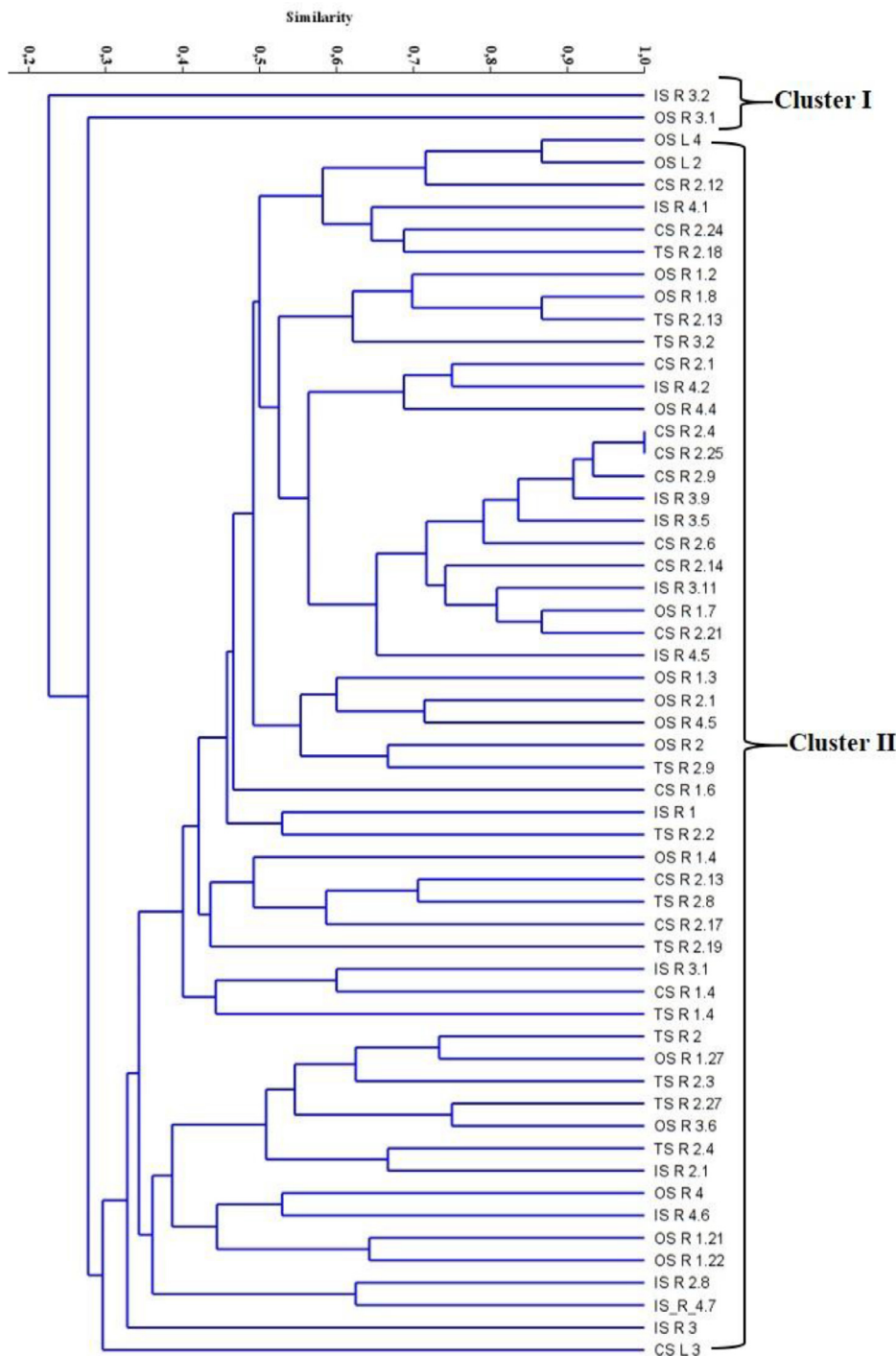


Fig. 5. Dendrogram obtained by the grouping carried out by the UPGMA (Unweighted Pair-Group Method with Arithmetical Average) algorithm from a similarity matrix between endophytic bacteria prospecting in different cocoa systems in the Brazilian Amazon. The Jaccard coefficient was used to construct the similarity matrix. CS L = Cabruca Systems Leaf; CS R = Cabruca Systems Root; TS R = Traditional Systems Root; OS L = Organic Systems Leaf; OS R = Organic Systems Root; IS R = Induced Systems Root.

as by [Etmiani and Harighi \(2018\)](#) and by [Qin et al. \(2011\)](#) and reviewed by [Garcia et al. \(2015\)](#), including systems involving cocoa plants ([Melnick et al., 2011](#); [Alsultan et al., 2019](#); [Ouattara et al., 2019](#)), which would be of great importance, considering that the main form of control for diseases and pests in cocoa crops is still the use of chemical pesticides.

Thus, crop matching experiments using 13 endophytic bacteria isolates that were selected for having three or more functional activities similar to that of biocontrol microorganisms were conducted against four phytopathogenic fungi ([Table 3](#)).

Among the isolates, the most promising were TS R 2.19, OS R 1.4, OS L 2, TS R 2.8 and CS R 2.24 with inhibition rates between 17.14%

and 50.72% ([Table 3](#)). We highlight isolate TS R 2.19, which presented superior antagonistic activity to the others, with inhibition rates between 25.07% and 50.72%, demonstrating antagonistic activity against all tested fungi. Similar results were found by [Shiomi et al. \(2008\)](#). Studying the selection of corn endophytic bacteria with antagonistic action against phytopathogens, they found inhibition rates ranging from 29.38% to 50.83%.

Percentages of inhibition of mycelial growth of pathogens of 40% or more are indicated by some studies as demonstrating possible potential as a biological control agent ([Lanna et al., 2010](#)). Work carried out by [Tullio \(2017\)](#), using cocoa endophytic bacteria with antagonistic potential for the control of fungi, demonstrated that isolates TCB 17 and TCB

Table 3

Inhibition of mycelial growth of phytopathogenic fungi by isolates of endophytic bacteria in vitro antagonism test.

Treatment	<i>Fusarium</i> sp.		<i>Cercospora coffeicola</i>		<i>Rhizoctonia solani</i>		<i>Pestalotiopsis guepinii</i>	
	R.C ¹ (cm)	I.R ² (%)	R.C ¹ (cm)	I.R ² (%)	R.C ¹ (cm)	I.R ² (%)	R.C ¹ (cm)	I.R ² (%)
Control	3,51 a	0,00	3,44 a	0,00	3,54 a	0,00	3,53 a	0,00
CS R 2.21	3,52 a	2,59	3,42 a	-9,55	3,51 a	1,52	3,38 a	5,53
TS R 2.19	1,78 d	50,72	1,76 e	43,35	1,78 d	49,94	2,68 c	25,07
OS R 1.4	2,46 c	28,75	2,08 d	40,38	2,94 b	17,14	2,78 c	21,42
TS R 2	3,21 b	7,05	3,50 a	-0,24	3,59 a	-1,17	3,13 b	11,50
OS L 2	2,64 c	22,87	2,17 d	38,53	2,83 b	19,00	2,65 c	21,19
TS R 2.8	2,49 c	27,13	2,46 c	30,26	2,42 c	30,70	2,73 c	18,84
CS R 2.4	3,42 a	-0,24	3,52 a	0,24	3,48 a	2,00	3,14 b	11,46
CS R 2.24	3,52 a	-3,17	2,79 b	20,92	3,24 a	8,80	3,38 a	4,41
CS R 2.9	3,39 a	5,67	3,43 a	2,43	3,61 a	0,00	3,54 a	0,93
CS R 2.25	2,93 b	18,52	3,56 a	-1,12	3,56 a	1,50	3,52 a	1,63
CS R 2.14	3,22 b	8,95	3,56 a	1,61	3,35 a	1,00	3,22 b	10,18
IS R 4.2	3,37 a	3,91	3,40 a	-1,81	3,60 a	-0,70	3,40 a	5,01
IS R 3.9	3,53 a	-0,59	3,28 a	2,06	3,60 a	-0,70	3,40 a	2,21
CV (%)	7,05		6,26		7,99		6,24	

*Means followed by the same letter do not differ statistically from each other, at the level of 5% probability by the Scott-Knott Test. 1 Average colony radius. 2 Relative inhibition according to [Shiomi et al. \(2008\)](#). CS R = Cabruca Systems Root; TS R = Traditional Systems Root; OS L = Organic Systems Leaf; OS R = Organic Systems Root; IS R = Induced Systems Root. CV (%) = Coefficient of Variation.

10 displayed a good reduction in mycelial growth of phytopathogens, highlighting TCB 17, which inhibited 86.38% the mycelial growth of *Rhizoctonia solani*.

Antagonism tests can indicate the potential for biological control of pests and pathogens and the use of endophytic bacteria may become an interesting strategy. There are few studies of endophytic bacteria in cocoa production systems in the Brazilian Amazon and understanding the interaction of these microorganisms with the host plant can provide alternative forms of disease control. In this work, five isolates of endophytic bacteria prospected in three of the four production systems showed potential for biological control, but more specific tests with in vivo inoculation are necessary to obtain more information about a possible biocontrol agent. Our results are pioneering in the main cocoa production region of Brazil.

4. Conclusions

Understanding the interaction between endophytic bacteria and cocoa plants is interesting to understand how communities of these microorganisms are established in different agricultural and/or agroforestry production systems. In this work, we conclude that endophytic bacteria colonize cocoa tree plant roots efficiently and that their population tends to increase in the rhizosphere, especially during the dry season. The functional and morphological characterization showed great variability, with endophytic bacteria playing important roles in the studied habitats, such as amylolytic, proteolytic and nitrogen fixing activities. These results help to understand the diversity of populations of endophytic bacteria in different cocoa production systems. Finally, the hypothesis that cocoa endophytic bacteria could perform antagonistic activities was demonstrated in the present study with isolates exhibiting promising growth inhibition rates. Our studies are incipient and the first in different cocoa production systems in the state of Pará, Brazil.

Declaration of Competing Interest

The authors declare no conflict of interest.

Author Contributions

Miguel Alves-Júnior: Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – original

draft, Writing – review & editing, Validation. **Fabiana Oliveira de Sousa:** Funding acquisition, Formal analysis, Data curation, Writing – original draft, Validation. **Thays Ferreira Silva:** Funding acquisition, Formal analysis, Data curation, Writing – original draft, Validation. **Ulisses Brigatto Albino:** Conceptualization, Methodology, Funding acquisition, Writing – original draft, Writing – review & editing, Validation. **Magali Gonçalves Garcia:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Validation. **Simone Maria Costa de Oliveira Moreira:** Formal analysis, Data curation, Writing – review & editing, Validation. **Marcos Ribeiro da Silva Vieira:** Formal analysis, Data curation, Writing – review & editing, Validation.

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