What is Inside of the Cacao Seed We eat? Approach to Mining the Cacao Microbiome (*Theobroma cacao*)

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DEDICATION

For all people in science, who have inspired me and transmitted me the admiration for the processes that articulate the complexity of the interactions of living organisms. With special admiration for my undergraduate professors Dr. Pedro Adolfo Jimenez and Dr. Liliana Franco.

I dedicate this work to my parents Elsa and Ricardo and my older small sister Catalina, my indefatigable motivators. To my friends Carmen Schlöder, Maria Fernanda Castillo, Diana Barrera, Cindy Celis, Julian Martinez and Francis van Oordt La Hoz, for the shared experiences and for being an example of courage, determination, and perseverance.

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ABSTRACT

Theobroma cacao, a native tree from the Amazon Basin, produces the principal commodity for preparing chocolate. In Latin America, cacao production is threatened by biotic and abiotic conditions that limit the survival of this crop. Endophytic bacteria are reported to support plant health by growth-promoting abilities, suppression of pathogens and pests, and detoxifying environmental contaminants. As part of a larger study on the microbiome of cacao lead by the INDICASAT- STRI (Panama), the global objective of this study is to characterize the bacterial microbiota-associated with cacao pods of two cacao varieties grown under field conditions in Panama (CATONGO and CATIE-R6) and also those associated with Peruvian seeds from the cacao variety CCN-51. Culture-dependent and independent methods were applied to isolate and characterize bacteria from seeds (Panama and Peru) and from three layers of the cacao pods (Panama varieties only). From the Peruvian seeds were isolated 53 morphotypes, which vary in morphology, being the genera *Bacillus* the most commonly found (12 different species). The isolates were subjected to a multitude of functional biochemical assays including growth promotion abilities and antimicrobial traits. About 47% percent of the isolates can synthesize molecules like indole acetic acid (IAA), siderophores, and organic acids adding to these traits, calcium solubilization. The top-performing isolates will be assessed in the future to test their ability as fertilizer agents. From the internal layers from the Panamanian pods, were isolated in total 160 morphotypes morphologically diverse, with the highest proportion found in the seeds (59 isolates). Interestingly the two evaluated varieties shared a low proportion of microorganisms in common (2-4 strains per layer), confirming that the microbiome is shaped based on the host genotype. *In silico* research, found that most of the strains (54 isolates) help the plant in the growth process.

RÉSUMÉ

Theobroma cacao, un arbre originaire du bassin amazonien, produit la composante principale de la préparation du chocolat. En Amérique latine, la production du cacao est menacée par des conditions biotiques et abiotiques qui limitent la survie de cette culture. La recherche suggère que les bactéries endophytes soutiennent la santé des plantes par leurs capacités de promouvoir la croissance, leur suppression de pathogènes et parasites, et leur détoxification de contaminants environnementaux. Partie d'une étude à plus grande échelle sur le microbiome du cacao menée par le INDICASAT- STRI (Panama), l'objectif global de cette étude est de caractériser le microbiome bactérien associé aux cosses de cacao de deux variétés de cacao cultivés à l'extérieur au Panama (CATONGO et CATIE-R6), et aussi le microbiome bactérien associé avec des graines péruviennes provenant de la variété de cacao CCN-51. Des méthodes dépendantes et indépendantes de la culture ont été appliquées pour isoler et caractériser des bactéries provenant de graines (du Pérou et du Panama) et de 3 couches de cosses de cacao (variétés panamiennes seulement). 53 morphotypes morphologiquement différent ont été isolés des graines péruviennes, avec le genre *Bacillus* étant le plus commun (12 espèces différentes). Les isolats ont été soumis à une multitude d'essais biochimiques, incluant les capacités de promouvoir la croissance et les traits antimicrobiens. À peu près 47% des isolats étaient capables de synthétiser des molécules tels l'acide indole acétique, ses sidérophores et des acides organiques, en plus de la capacité à solubiliser le calcium. Les isolats les plus performants seront évalués plus tard pour leur capacité d'agir comme engrais. 160 morphotypes morphologiquement diversifiée ont été isolés des couches intérieurs des cosses panamiennes, avec la plus grande proportion dans les graines (59 isolats). D'intérêt particulier, les deux variétés évaluées ont partagé un faible nombre de microbes en commun (2 à 4 souches par couche), confirmant que le microbiome est formé en accord avec le génotype de l'hôte. La recherche *in silico* a trouvé que la plupart des souches (54 isolats) aident la plante dans le processus de croissance.

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CONTRIBUTION OF AUTHORS

This work has been written in the traditional thesis format as per the Department of Plant Science and Graduate studies at McGill University according to “Guidelines for Thesis Preparation and Submission”. The work herein was done under the supervision of Professor Jabaji and co-authorship of Drs. Herre and Mejia and with financial support from McGill-STRI NEO (Neotropical Environment Option) and BESS (Biodiversity, Ecosystem Services, and Sustainability, and the Natural Sciences and Engineering Research Council of Canada (NSERC). The candidate conducted the field collection, isolation and subsequent characterization studies. Data compilation, and analysis was also done by the candidate. This thesis was written by the candidate under the supervision of Dr. Jabaji, with special help from Nathan Liang for the French abstract, and is composed of 8 chapters.

The author, Ximena Florez-Buitrago, performed the experiments detailed below, data analysis and thesis writing. Dr. Jabaji provided funds and support for the research, including supervisory support and thesis review.

Chapter 1

1. Introduction

Plants are hosts of complex communities of endophytic bacteria and fungi that colonize the interior of below and above ground tissues (Nazir & Rahman, 2018). Bacteria living inside plant tissues are acquired from the environment with each new generation and are most likely transmitted horizontally as endophytes. Accumulating evidence indicates that seed endophytes have beneficial effects (Frank et al., 2017). To name a few examples, seed endophytes promote seed germination through the production of phytohormones, and some endophytes have antimicrobial properties (Shahzad et al., 2018). As a global strategy to reduce chemical fertilizers and pesticides, some endophytes and rhizospheres' bacteria can be used for plant growth promotion and health (Scott et al., 2018). Consequently, a better understanding of bacterial endophyte transmission routes and modes will benefit studies of plant–endophyte interactions in both agricultural and natural ecosystems (Glick, 2020).

Cacao (*Theobroma cacao*) is a tropical crop with an ancient relation to humankind (Coe & Coe, 2003). Chocolate consumption continues growing around the world annually (Statista, 2020). In the last year, 4.85 million tonnes of cacao were produced in total (Altendorf, 2017), with 16% of the total being provided by the Latin American countries. This business employs around two million people, generating profits close to one hundred millions dollars per year for chocolate manufacturers (Arvelo-Sánchez et al., 2017).

In Latin America, cacao production is challenged by several abiotic and biotic factors, among them the frosty pod rot disease caused by the fungus *Moniliophthora roreri*, which generates yield losses over 30%, but could reach 100% in some circumstances (Barbosa et al., 2018). Pesticide application costs are beyond the economic means of small-scale growers. Moreover in times of climate change it is impossible to ignore the serious ecological impacts (Evans, 2016). Additionally, the large numbers of consumers and farmers interested in either ecologically-based pest

management or organic farming have led to an increased interest in biological control options for the management of cacao diseases.

Biopriming is an example of a practical technique, in which seeds are coating with live cells. Researches have been used endophytic bacteria that exhibit traits of agronomic interests, getting exceptional results (Ozbay, 2018). For instance, Rivarez (et al., 2019) applied this technique to induce resistance in a variety of papaya (*Carica papaya*) susceptible to the disease Bacterial Crown Rot (BCR) caused by the bacterium *Erwinia mallotivora*. He coated the seeds with a *Bacillus* strain isolated from a tolerant papaya variety to that disease. As an overall result, it was improved the percentage of survivance of the plants in the field. However, seed biopriming requires a careful design that includes the osmotic potential of the seeds; CFU concentration; incubation time, environmental factors, such as temperature and soil type (Ozbay, 2018).

In this thesis, the potential of endophytic bacteria isolated from cacao seeds and fruits of three different varieties of cacao from three Latin American countries was explored. Chapter 3 describes the bacterial community associated with organic, fermented and dried Peruvian seeds. Diversity of bacterial morphotypes were isolated using culture-dependent methods and characterized by culture-independent method applying partial 16s rRNA sequencing. The isolates belonged to several taxonomic groups with the majority belonging to *Bacillus* representing 12 different species. The isolates were screened for plant growth promotion abilities as well as plant colonization potential. About 47% of the isolates exhibited growth promotion potential through calcium solubilization and the production of molecules such as: indole acetic acid (IAA), siderophores, and organic acids adding to these traits.

Recent literature demonstrates that plant genotype can modulate the composition of the microbiome in the rhizosphere and roots, and in the phyllosphere, indicating that the host innate immune system and root metabolites mainly shaped root microbial community structure (Compant et al., 2010). Chapter 4 describes the bacterial microbiome of two cacao varieties: CATONGO and CATIE-R6 based on their tolerance to frosty pod rot disease. We explored the diversity of the microbiome associated with different layers of cacao fruits (i.e., endocarp, pulp, and seeds). Partial 16S rRNA sequencing identified a total of 160 endophytic bacterial morphotypes morphologically

diverse, exhibiting contrasting differences between the two varieties of cacao, in terms of microbial community composition. The composition of each layer was unique with a low proportion of bacterial species common to the three layers.

1.1. Objectives

- Isolation, identification and biochemical characterization of culturable bacterial endophytes associated with Peruvian cacao seeds, variety CCN-51.

- Description of the diversity of the bacterial microbiome of two Panamanian cacao varieties CATONGO (susceptible) and CATIE-R6 (tolerant) with different tolerance levels to the frosty pod rot disease.

1.2. Hypothesis

- Cacao fruit-associated bacterial communities are taxonomically diverse.
- The composition of bacterial communities of cacao layers are tissue-specific.
- Bacterial strains associated with cacao fruits exhibit functional biochemical attributes that can potentially be used as fertilizers.

Chapter 2

2. Literature Review

2.1. Morphological Description of Theobroma cacao

The chocolate tree is a woody species, belonging to the family *Malvaceae,* genus *Theobroma,* species *Theobroma cacao L*. It can reach a height of up to four to eight meters (Batista, 2009). Its leaves are alternate and deciduous, usually every two or three months budding peak of new leaves appear (Batista, 2009). One individual flower is hermaphrodite and is supported by a strong pedicel, the flowers are grouped into specialized structures called ¨floral cushions¨. These are located directly on the trunk of the tree. Typically, cacao flowers develop directly on the trunk and mature branches. The flower opens 20 to 25 days after the appearance of a tiny floral button (Thi et al., 2016).

The process of pollination in cacao is challenging because the pollen is viable only for three days, the floral structure prevents the pollen from being easily reached by insects, and the flowers are only receptive during the first hours of the morning. Studies confirm that insects from the genus *Forcimpoyia spp* are the most successful natural pollinators of these flowers due to their small size (1-3 mm in length), (Soria, 1971). If the flowers are not successfully fertilized, they fall three days later (Thi et al., 2016).

Fruits or pods are indehiscent berries that can vary in shape, thickness, roughness, color, and size depending on their genetic origin. A whole range of sizes shapes and colors are observed, in an immature stage they can exhibit green, reddish and brown tones; while in the mature stage, they vary from yellow, yellowish-brown to orange-reddish **(Figure 2.6.1)**, (Ramsey, 2016). Some pods are smooth and round **(Figure 2.6.1 B)**, others have deep grooves **(Figure 2.6.1 A)**, (Batista, 2009).

The hard-outer shell (exocarp) of the pod is technically an ovary, contributing to 70% of the total weight (Ramsey, 2016). It plays a protective role, followed by a slightly woody layer called mesocarp, and finally, the endocarp surrounding the seeds **(Figure 2.6.2)**. The seeds are organized in rows, around a central axis called the placenta, in

which the seeds are covered with the pulp, a mucilage rich in sugars. The maturation of the fruit occurs between 150 and 180 days after the pollination, however, this process relies on the genetic pool and weather conditions (Ramsey, 2016).

2.2. Cacao Fermentation Process

Obtaining chocolate is a long and rigorous process, which begins with the selection of healthy and mature fruits in the field. Fruit pods are open using a machete to be able to cross the strong exocarp that protects the seed which is collected and immediately fermented inside wooden boxes covered with plantain leaves. This initial step is crucial because the microbes use the sugar from the pulp to produce ethanol and acetic acid, which is absorbed by the embryo, killing it and causing the production of flavor precursors (Ramsey, 2016).

At the beginning of the fermentation process, the environment presents low oxygen levels and an acidic pH in the pulp (around 3.6). In these conditions, the yeasts can colonize and metabolize the carbohydrates present in the pulp. The size population of the yeast starts to increase during the first 12 hours, generating also the conditions to support the development of lactic acid bacteria (LAB), (Schwan & Wheals, 2004).

Once the group of lactic acid bacteria reaches the exponential phase (36 hours), the yeast population starts to diminish. As time passes, the temperature rises above 37ºC, reducing the concentration of ethanol, and lactic acid (AAB), creating, a suitable environment for the development of acetic acid bacteria. After 88 hours, they are the most predominant microorganisms in the fermentation boxes. As a result of the biochemical reactions performed by AAB, a high temperature (up to 50ºC) is registered, which causes the gradual decline of this group of bacteria (Schwan & Wheals, 2004). After 120 hours of fermentation, acetic acid bacteria are absent.

At the end of the fermentation, the beans are transferred to platforms and sun-dried. During this stage, (after 156 hours), there is an abrupt loss of the total microbial population. Only microorganisms that can form spores, like the genus *Bacillus* and filamentous fungi, can survive (Schwan & Wheals, 2004).

The seeds are then packed into breathable burlap sacks to be exported to chocolate manufactures (Ramsey, 2016). The fermentation process must be done once the pods

are open because of that, it is impossible to get fresh cacao fruits in non-producer countries. Moreover, in the case that farmers wanted to export fresh cacao pods, the production costs will rise dramatically, the average weight of one pod is 400 g. Each pod contains 50 seeds, and about 400 dried cacao seeds are required to make 450 g of chocolate (Cargill, 2020).

2.3. Life Cycle Moniliophthora roreri

Studies performed in cacao farms in Ecuador had indicated that the spores of the fungus *M. roreri* can disperse over an area of one kilometer and persist throughout the year (Ploetz, 2016). The highest frequency of the disease occurs during the rainy season due to the high humidity and the increase of the velocity of the winds. Spores that are released during the first few days of sporulation are more vulnerable to abiotic factors because their cell walls are thin (Evans, 2016). However, with time the spores' structure is strengthened, surviving for up to nine months on hanging pods. Once those spores reach the soil they can be infectious during 30 days. (Bailey & Meinhardt, 2016). Infected pods rapidly lose moisture following fungal colonization, becoming mummified. Gradually, the spore bloom is eroded by weathering and ultimately only the flaking pseudostroma persists **(Figure 2.6.3)**, (Bailey & Meinhardt, 2016).

The spores germinate and penetrate the pod epidermis, or via stomata, infecting the tissues intercellularly without causing necrosis. The pods are most susceptible to infection during the initial three months of development. The entire process from penetration to intercellular colonization and intracellular invasion (necrosis) occurs within 40 days. Once the infection occurs it leads to different phenotypic symptoms including premature ripening, pod deformation before visible necrosis **(Figure 2.6.4)**, (Evans, 2016).

2.4. Cultural and Biological Control of *Moniliophthora roreri*

The regular harvesting and the weekly removal of infected and mummified pods reduces the inoculum sources. As well as, reduction of the tree height (four meters) and diminution of the humidity by the Increase of the brightness combined with fungicide applications can reduced the disease by 90% (Ten Hoopen & Krauss, 2016).

Monthly applications of a copper fungicide (Kocide) were not very effective reporting yield losses above 75%, (Ten Hoopen & Krauss, 2016). Other results employing a systemic fungicide (Oxathiiin flutolanil), improved total pod production at early stages but was less effective at the highest point of production. One of the reasons could be related to the fast-longitudinal expansion of cacao pods (3 mm per day), (Ten Hoopen & Krauss, 2016). Thus, the use of antagonistic microorganisms naturally found in the phyllosphere, rhizosphere, soil, and internal tissues of the fruits is an attractive approach **(Figure 2.6.5).**

Evans and collaborators (2003), isolated mycoparasitic fungi belonging to the genera *Clonostachys* and *Trichoderma.* Experiments showed that some of these biocontrol agents could survive up to 3 months in/on cacao pods and could partly control the sporulation of *M. roreri*. Likewise, fungal endophytic colonization of cacao leaves reduced their susceptibility to *Phytophthora palmivora* (Arnold & Herre, 2003).

Another successful example was based on the use of fungi species like *Trichoderma stromaticum*, *T. theobromicolal,* and *T. paucisporum.* These species produced a diffusible antifungal that inhibited the development of *M. roreri* in vitro and on-pod trials (Samuels et al., 2000; Samuels et al., 2006). The application of foliar sprays of a *T. martiale* strain on the pod (30-40 days post-application), showed a clear-cut control of black pod diseases of cacao caused by *Phytophthora palmivora* (Ten Hoopen & Krauss, 2016).

Despite promising results in using fungal endophytes to inhibit or suppress cacao disease, research on the diversity and presence of bacterial endophytes is very limited. Besides, accumulated evidence suggests that the disease reduction by using endophytes could be due to the combination of antimicrobial activities and growth stimulation attributes (Yánez-Mendizábal et al., 2012).

Actinomycetes were reported to be common inhabitants of the cacao rhizosphere and pod surfaces. Some of these isolates were reported to exhibit antagonistic activity under laboratory conditions against black pod and witches' broom pathogens (Melnick et al., 2011).

Similarly, two isolates of bacterial endophytes (*Bacillus subtilis* and *Enterobacter cloacae)* associated with different organs of healthy cacao trees were reported as

excellent colonizers of cacao seedling, exhibiting not only growth promoter capabilities (Leite et al., 2013), but antimicrobial activities against cacao pathogens (Falcäo et al., 2014), thus making them excellent biological control agents. Experiments performed using bacteria of the genus *Bacillus,* demonstrated that these microorganisms are capable of colonizing cacao leaves, primarily as epiphytes but also as endophytes. Their presence led to a significant decrease in disease severity when the leaves were inoculated with *Phytophthora capsica* (Melnick et al., 2008) or with *M. roreri* and *M. perniciosa* (Melnick et al., 2011). These results strongly suggest that the bacteria either induce systemic resistance or act directly on the pathogen.

2.5. General Mechanisms Employed by Plant Growth Promotion Bacteria in Plant Protection

There are a group of microorganisms known as plant growth-promoting bacteria (PGPB) that have been forming symbiotic/mutualistic relationship with flowering plants for the last 80-100 million years. They are recognized to be able to interact and provide a range of benefits to the host plant, using direct and indirect mechanisms (Figueiredo et al., 2016), **(Figure 2.6.6)**.

As a consequence of treating plants with PGPB one or more of the following effects may be observed in the host: (i) increased plant biomass; (ii) increased plant nitrogen, phosphorus and iron content; (iii) increased root and/or shoot length; (iv) enhanced seed germination; (v) increased plant disease resistance; (vi) increased plant tolerance to various environmental stresses; (vii) increased production of useful secondary metabolites; and (viii) better plant nutrition, especially the edible portions of the plant (Glick, 2020).

PGPB are classically found in the soil area immediately around the roots. This location reflects the fact that the plant roots exude from ~5% to ~30% of the carbon that is fixed through photosynthesis, and provide this fixed carbon as a source of food to soil microorganisms (Glick, 2020). Moreover, the root exudates modify the chemical and physical properties of the soil and regulate the bacterial community that is present in the area surrounding the root surface (Jacoby et al., 2017). Root exudates can chemoattract or chemo repels specific bacterial strains toward the root (Saleh et al., 2020). Also,

quorum-sensing molecules are increased likewise with the exudates in the presence of bacteria in the rhizo- and endosphere (Chaparro et al., 2013). Quorum sensing molecules is a group of molecules responsible for cell-to-cell communication between plants and bacteria. They allow bacteria to share information about their cell density and regulates the expressions of various genes (mainly in the roots) linked to plant development (Zhou et al., 2016).

Alternatively, some PGPB named endophytes can colonize an internal portion of the plant tissues. The endophytic bacterial population is variable in different plant organs and tissues and are shown to vary in their abundance as low as hundreds to as high as $9 \text{ X } 10^9$ of bacteria per gram plant tissue (Puri et al., 2018). Endophytes can be classified into two types; type A (facultative) are bacteria that are bound relatively non-specifically to plant surfaces and they enter into the plant through wounds, stomata, lenticels, root cracks, and germinating radicles. Type B (obligate) are bacteria that colonize only a narrow-limited group of plant species, they are referred to as symbiotic bacteria because following the infection the bacteria typically form nodules on the plant roots (Glick, 2020), and type C (facultative or obligate) is the avirulent forms of plant pathogens. Researchers have described sophisticated mechanisms of physiological regulation (i.e., ethylene), which are articulated among the interaction between the host plant, bacterial endophytes and the microorganisms from the rhizosphere. The development of these systems has been the key to enhancing traits that assure their selection and be a part of the regulation process (Hardoim et al., 2008), **(Figure 2.6.6)**.

2.5.1. Direct Mechanisms

Endophytes that are not nitrogen fixers exhibit traits such as the production of siderophores, cellulases, and phosphatases, phytohormones.

- *Siderophores Production*

Siderophores are biogenic organic ligands with high affinity and specificity for binding iron, they are induced at low iron concentrations for biological assimilation of Fe^{3+} (Hersman, 2018). Over 500 siderophores have been characterized by culturable bacteria. Although they are structurally different these molecules have in common the small low

molecular mass (0.5 – 1.5 kDa) and their metal-binding groups: αhydroxy carboxylate, hydroxamate, catecholate, and carboxylate groups (Kraemer et al., 2018).

Hydroxamate-type siderophores are the most common siderophore type in nature, being widely distributed in bacteria and fungi (Årstøl and Hohmann-Marriott, 2019). This type of siderophore can be detected using the chrome azurol (CAS) agar assay (Louden et al., 2011).

The siderophores are synthesized by non-ribosomal peptide synthetases (NRPSs) or polyketide synthase (PKS) domains that work in concert with NRPS modules. Siderophore synthesis via NRPSs is the most common form and consists of a multienzyme assembly line, in which amino acids, as well as carboxy and hydroxy acids, are built into a peptidic precursor molecule, which is subsequently modified by either NRPSs or other enzymes to form the final siderophore (Kramer et al., 2020).

The uptake of iron in Gram-negative bacteria involves the specific recognition of the iron-loaded siderophore by a β-barrel receptor in the outer membrane. After binding the ligand, the receptor undergoes a conformational change, translocating the iron-loaded siderophore into the periplasm. This process is supported by a TonB complex, which delivers the energy via the proton motive force (Kramer et al., 2020). Transport of the iron-loaded siderophore into the cytosol, where iron reduction occurs, is mediated by an ATP-binding cassette transporter in the inner membrane. In some special cases, the iron is reduced in the periplasm and only the ferrous (Fe2+) iron is imported into the cytosol. In Gram-positive bacteria, no outer-membrane receptors are required, and siderophores are directly imported by ATP-binding cassette transporters spanning the cell membrane. The fate of siderophores after the release of iron is not well understood. In some cases, siderophores can be recycled through specific recycling mechanisms whereas other siderophores undergo hydrolysis to release iron (Kramer et al., 2020).

Iron (Fe) is the sixth most abundant element in the universe and the fourth most abundant in the earth's crust. However, Fe availability is conditioned by the soil redox potential and pH. In aerobic soils or of higher pH, Fe is readily oxidized and is predominately in the form of insoluble ferric oxides. At lower pH, the ferric Fe is freed from the oxide and becomes more available for uptake by roots (Morrissey & Guerinot, 2009).

Iron is an essential nutrient for plants, and its deficiency is exhibited in severe metabolic alterations, mainly because iron is present as a cofactor in many enzymes essential to physiological processes, such as respiration, photosynthesis, and nitrogen fixation (Taiz & Zeiger, 2010). During iron-limiting circumstances, plant growth-promoting bacteria and fungi can secrete and released siderophores into the rhizosphere soil (Miethke & Marahiel, 2007).

Although the role of siderophore biosynthesis by endophytes in plant colonization is unknown, some evidence proposed that these compounds play a role in the induction of host Induced Systemic Resistance (ISR), as well as in the biocontrol process by diminishing the availability of iron to pathogens (Afzal et al., 2019).

- *Indole acetic Acid (IAA)*

Phytohormones are chemical messengers involved in a broad spectrum of physiological and biochemical processes of higher plants at very low concentrations. Conventionally, phytohormones consist of five classes—auxins, abscisic acid, cytokinin, gibberellins, and ethylene—as well as their precursors and synthesized analogs (Xingfeng et al., 2018).

The auxin indole 3-acetic acid (IAA) can promote several growths and developmental events such as cell division, elongation, and differentiation (Egamberdieva et al., 2017). Besides, auxins have a crucial role in promoting heavy metal tolerance like lead (Pb) and in the mitigation of salt stress (Egamberdieva et al., 2017).

Researches have suggested that around 80% of the microorganisms from the rhizosphere can synthesize IAA. Scientists have characterized species able to release this hormone during pathogenic and symbiotic scenarios (Gamalero & Glick, 2015). Some explanations provide an understanding as to why bacteria would synthesize a plant hormone. (i) through the stimulation of the plant growth, the bacteria can also increase the production of plant metabolites that they can use for their survival. (ii) another reason can be explained from the perspective of the detoxification of tryptophan analogs, which are harmful to bacterial cells. (iii) inhibition of the plant defense enzymes (i.e. chitinase, β-1,3-glucanase) to facilitate the process of bacterial colonization (Patten & Glick, 1996).

Besides, based on the auxin available in the environment can be registered three different scenarios: (i) bacterial IAA genes can be transferred directly into the host plant genome (i.e. Agrobacterium species); (ii) the infecting bacterium can live within the plant tissues where it can secrete IAA into surrounding tissues; or (iii) the bacterium can live outside of the host plant, colonizing its surface. Commonly, the bacterial phytopathogens colonize the plant tissues and/or transfer its IAA gene to the host, stimulating the plant growth to the point of gall formation. In the case of PGPB, an increase of metabolites as opines is registered, they exert their effect colonizing the external surface of a plant (Patten & Glick, 1996).

Bacteria use different biosynthesis pathways; indeed, a single strain can synthesize IAA in more than one pathway. There are five different pathways, but the pathway indole-3-acetamide is constitutive in most of the microbes. The enzyme nitrile hydratase (induced by indoleacetamide) catalyzes the initial step in the conversion of indole acetonitrile to IAA (Kobayashi et al., 1995). Experiments performed with exogenous radiolabelled tryptophan shown that this amino acid was converted to IAA, through the intermediate indoleacetamide. Likewise, the gene for tryptophan 2-monooxygenase (iaaM) was identified and isolated. It was transferred and tested in new clones using Salkowski's chemical assay (Patten & Glick, 1996).

2.5.2. Indirect Mechanisms

Organisms can exert pathogen control, through four key mechanisms: (i) parasitism, (ii) antibiosis, (iii) competition for resources, and (iv) induced resistance.
The plants maintain a natural relationship with many endophytes and epiphytes, some of these microbes can provide at least some protection from disease attacks however, the impact on these microorganisms on disease reduction remains unknown (Arnold & Herre, 2003; Herre et al., 2007; Tchameni et al., 2011).

- *Lytic Enzymes*

Endophytes are present in the seeds. It has been documented that during the germination they degrade the cellulose of the cuticule and make carbon available for the plant germination and establishment (Jain & Pundir, 2017). Industrially the cellulases are attractive molecules due to their massive applicability in various processes such as biofuels like bioethanol, triphasic biomethanation; agricultural and plant waste management; chiral separation, and ligand binding studies (Gupta et al., 2012).

- *Hydrogen cyanide (HCN)*

Cyanide is a secondary metabolite produced by some gram-negative bacteria. Hydrogen cyanide (HCN) is formed from glycine, which is one of the predominant amino acids of the root exudates (Lesuffleur et al., 2007). The glycine is oxidized to iminoacetic acid [H-C(NH)-COOH]. Then, the C—C bond is split, with a concomitant second dehydrogenase reaction, which produces HCN and $CO_{2)}$, (Laville et al., 1998).
No HCN is produced under fully anaerobic conditions when nitrate is the terminal electron acceptor, the optimal expression of HCN synthase occurs during the transition from the exponential to the stationary phase and at low oxygen levels (Laville et al., 1998).

HCN is a volatile, secondary metabolite that suppresses the development of microorganisms and also directly promote plant growth by increasing root hairs (Luz, 2001). HCN is one of the significant inhibitors of cytochrome C oxidases and other metalloenzymes which are involved in the metabolic activities of phytopathogens (Jaiswal et al., 2019)

- *ACC Deaminase Activity*

ACC deaminase (1-aminocyclopropane-1-carboxylate deaminase) is a multimeric enzyme, cytoplasmically localized, that utilizes the coenzyme pyridoxal phosphate as a cofactor (Singh et al., 2015).

ACC deaminase is induced in the presence of the substrate ACC, which is the immediate precursor of ethylene in plants. After cleaving this molecule, the products generated are ammonia and α-ketobutyrate. This enzyme encoded by the gene AcdS, it is regulated by the gene *AcdR* (Singh et al., 2015).

ACC deaminase activity has been registered in numerous gram-positive and negative rhizobacteria. Evidence suggests that some bacteria are only able to produce the enzyme when the bacteria is localized inside of a root nodule (symbiotic phase), (Gamalero & Glick, 2015). Most of the microorganisms isolated that producing ACC deaminase have a basal activity of the production of this enzyme. However, ACC deaminase synthesis is induced by a minimum concentration of 100 nM of ACC at 30ºC pH:8.5, requiring up to 10 h of induction (Gamalero & Glick, 2015). Several amino acids like $_{L}$-Ala, $_{DL}$-Ala, and $_{DL}$-Val γ- aminoisobutyric acid can induce activity to almost the same level as ACC (Singh et al., 2015). Nevertheless, ACC deaminase is not as widely spread among endophytic bacteria as previously thought (Hardoim et al., 2015).

Phylogenetic analysis based on sequences of the gene *AcdS* revealed that the ancient ancestors belong to the phyla *Actinobacteria* and *Deinococcus-Thermus*, which present this gene in their primary and unique chromosome (Singh et al., 2015). In comparison with most *Burkholderia* and *Cupriavidus spp*. strains, this gene is located on a second smaller chromosome, while in other *β-Proteobacteria* it is located on the primary chromosome or megaplasmids (Singh et al., 2015). This evidence suggests that the *AcdS* gene has been transmitted vertically mostly and occasionally through horizontal gene transfer.

During vertical transmission, limitations, such as adaptation to specific niches, induced *AcdS* divergence or gene loss. The advantages conferred by ACC deaminase activity have been positively selected by evolution, leading to intragenomic transfers of *AcdS* genes from primary chromosomes to plasmids and increased divergence of *AcdS* genes (Nascimiento et al., 2014).

Endophytic bacteria that synthesize the enzyme ACC deaminase facilitate plant development ensuring that ethylene levels stay below the point where plant-growth is impared. Likewise, the ACC deaminase help the plant to survive under abiotic stress such as drought, salinity, temperature, waterlogging, and presence of heavy metals. (Glick, 1995).

- *Organic Acids*

Organic acids are broadly produced by some species belonging to different kingdoms (animal, plant, and microbes). Also, there is a great variety of organic acids, they can contain one or more carboxylic acid groups, which may be covalently linked in groups such as amides, esters, and peptides. Only from the microorganisms have been isolated more than 100 acid compounds. They range from simple, unsubstituted acids (i.e. formic), to complex, glycosylated acids (I.e. such as pyolipic acid). The most complex acids containing branch chains (i.e., isovaleric acid). More than a dozen di- and tricarboxylic acids are known, while the number of known hydroxy- (or keto-) acids of microbial origin exceed 50. Commercially the most important are: citric, gluconic, itaconic, ascorbic, and lactic acids (Papagianni, 2019).

Microbes can produce organic acids using two different pathways, the main ones used by aerobic microorganisms are the tricarboxylic acid cycle (TCA) glycolysis, and those derived from the direct oxidation of glucose. As a result, from those processes are produced citric, lactic, itaconic, and malic acids. In the specific case of the gluconic and acetic acids they are produced by one or two enzymatic steps from glucose (Papagianni, 2019).

A common quantitative test for doing the detection of the production of organic acid by bacteria is using the methyl red method. Some bacteria have the ability to convert glucose into pyruvic acid, which is further metabolized to produce a stable acid, mainly lactic and acetic acid. The acid produced decreases the pH to 4.5 or below, which is indicated by a change in the color of methyl red from yellow to red (Dalynn Biologicals, 2002).

In the case of the plant root exudates are classified into two categories: low molecular weight (amino acids, organic acids (OAs), sugars, phenolic compounds), and high molecular weight compounds (polysaccharides and proteins), (Macias-Benitez et al., 2020). Most of the low molecular weight compounds have been hypothesized to present a functional significance in regulating ecosystem productivity. Low molecular weight carboxylic acids (LMWOAs) play a significant role in the rhizosphere as essential factors for nutrient acquisition, mineral weathering, and alleviation of anaerobic stress in roots (Macias-Benitez et al., 2020).

The LMWOAs are characterized by a mass from 46 to 100 Daltons. Of the wide array of OAs present in the soil, the LMWOA such as oxalic, citric, malic, acetic, formic, and tartaric acids have been identified to play significant roles in soil productivity. The efficiency of OAs for soil biological processes follows a decreasing order from tricarbolic LMWOAs (citric and tartaric acids), dicarboxylic LMWAOAs (maleic and fumaric acids), and the monocarboxylic LMWOAs (oxalic and acetic acids) as the least (Adeleke et al., 2017). Recent evidence points out that organic acids exuded from plant roots play an important role in selective recruitment and colonization of PGPR and inducing biofilm (Saleh et al., 2020).

The average total content of these acids is estimated as 10% of the total dissolved organic carbon (Van Hees & Lundström, 2000). In a wide range of soils, the concentration of these products in soil solution ranges from 0 to 50 μM for di/tricarboxylic acids (oxalic, malonic, malic, succinic, tartaric, and citric acid) and from 0 to 1 mM for monocarboxylic acids (formic, acetic, propionic, butyric, valeric and lactic acid). However, the concentrations can change depending on soil type and environmental conditions (Adeleke et al., 2017).

2.6. Figures

Figure 2.6.1. Stages of development of cacao fruits in two commercial varieties. Pod maturation is accompanied with changes in size and coloration pattern of the pod. **A.** Variety ICS-95 T1 **B.** Variety CATIE-R4 (Phillips, 2012).

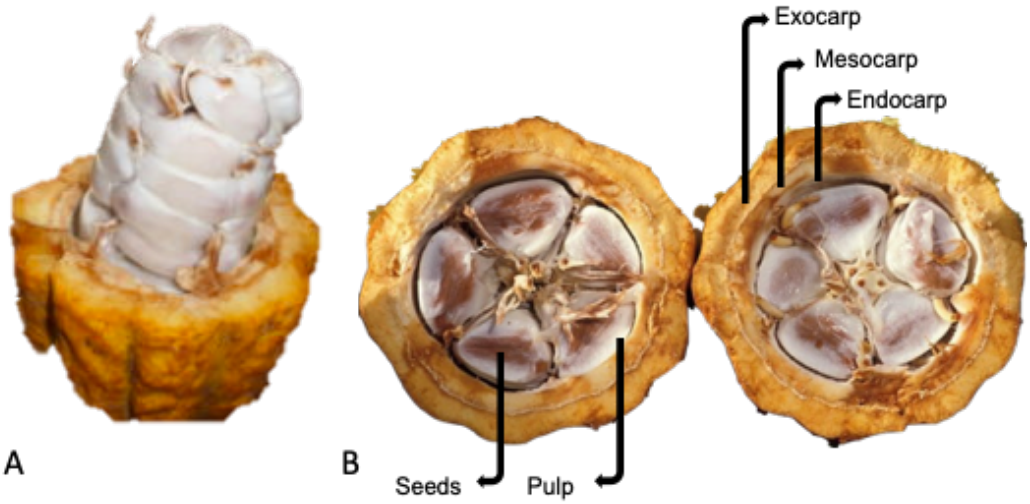

Figure 2.6.2. Dissection of the mature cacao fruit. A. View of the central axis and location of the seeds. B. Transversal dissection, exhibiting the different layers of the pod. (United States Department of Agriculture, 2018)

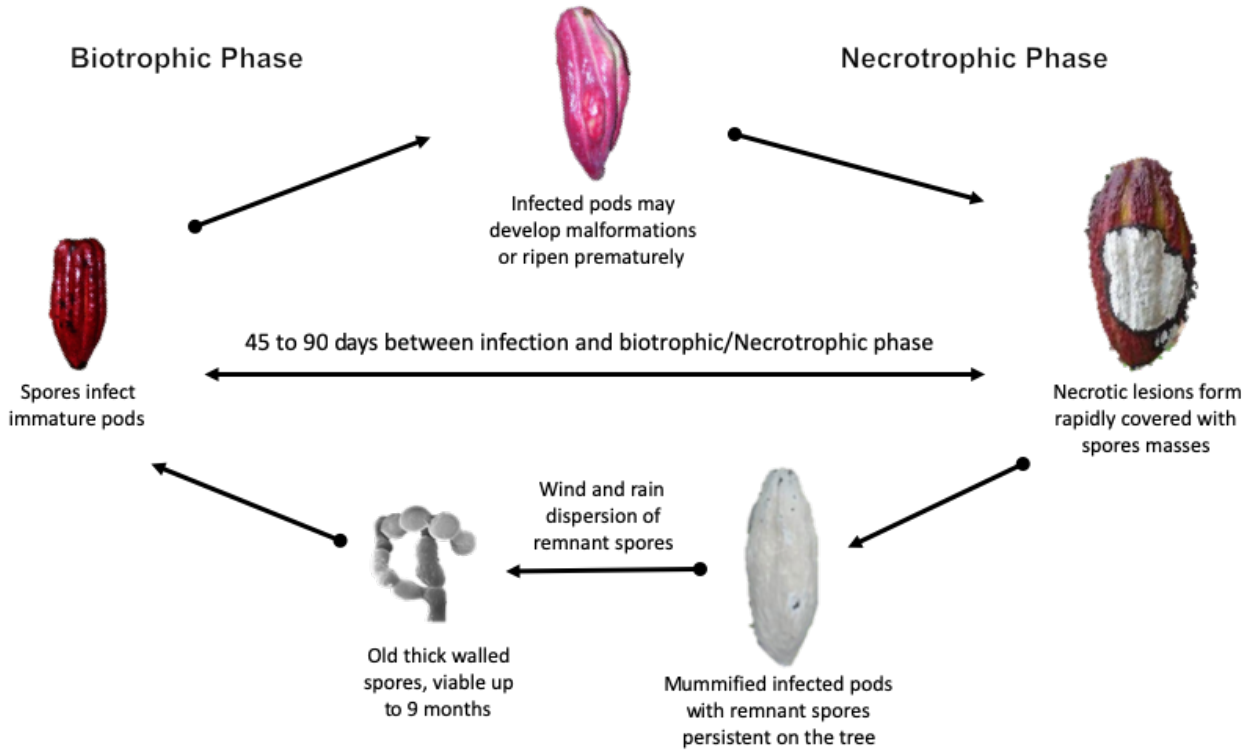

Figure 2.6.3. *Moniliophthora roreri* disease cycle on *Theobroma cacao*. Spores produced on necrotic pods infect new pods, initiating a prolonged biotrophic phase (45–90 days). Once the necrotrophic phase is initiated, new spores are rapidly produced on pods and spread by wind and rain (Bailey et al., 2018).

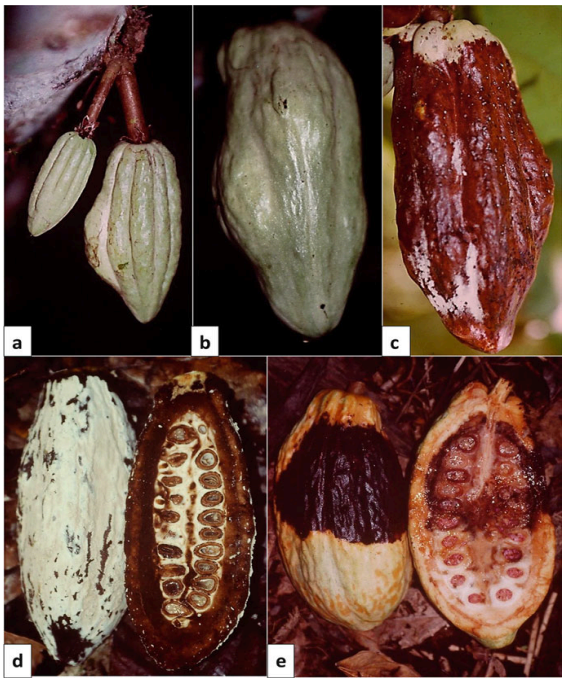

Figure 2.6.4. Symptoms of *Moniliophthora roreri* infection (a) Young pod (~1-month-old) with gross swelling. (b) Swollen and deformed green pod, 2–3 months old. (c) Pod, 3–4 months old, showing swellings and initial phase of necrosis. (d) Pod with developing pseudostroma, internal necrosis with compaction and complete destruction of the bean mass. (e) Atypical black external lesion—more typical of infection by *M. perniciosa* (Evans, 2016).

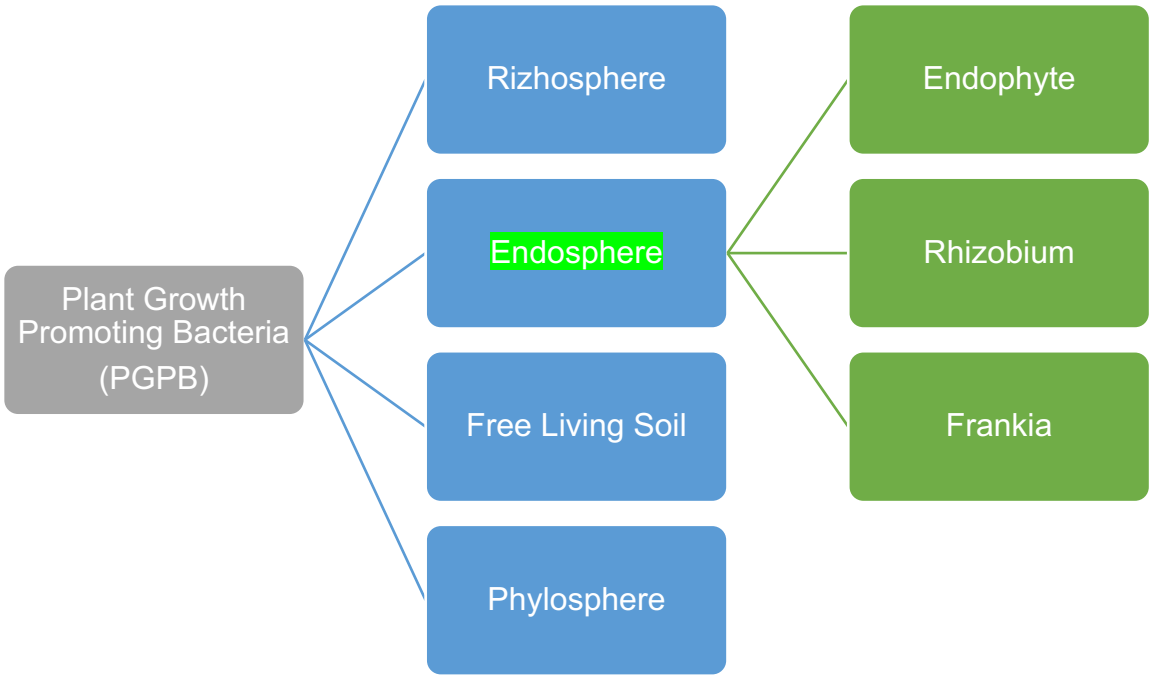

Figure 2.6.5. Categorization of plant growth-promoting bacteria (PGPB) based on their different ecological niches (Puri et al., 2018).

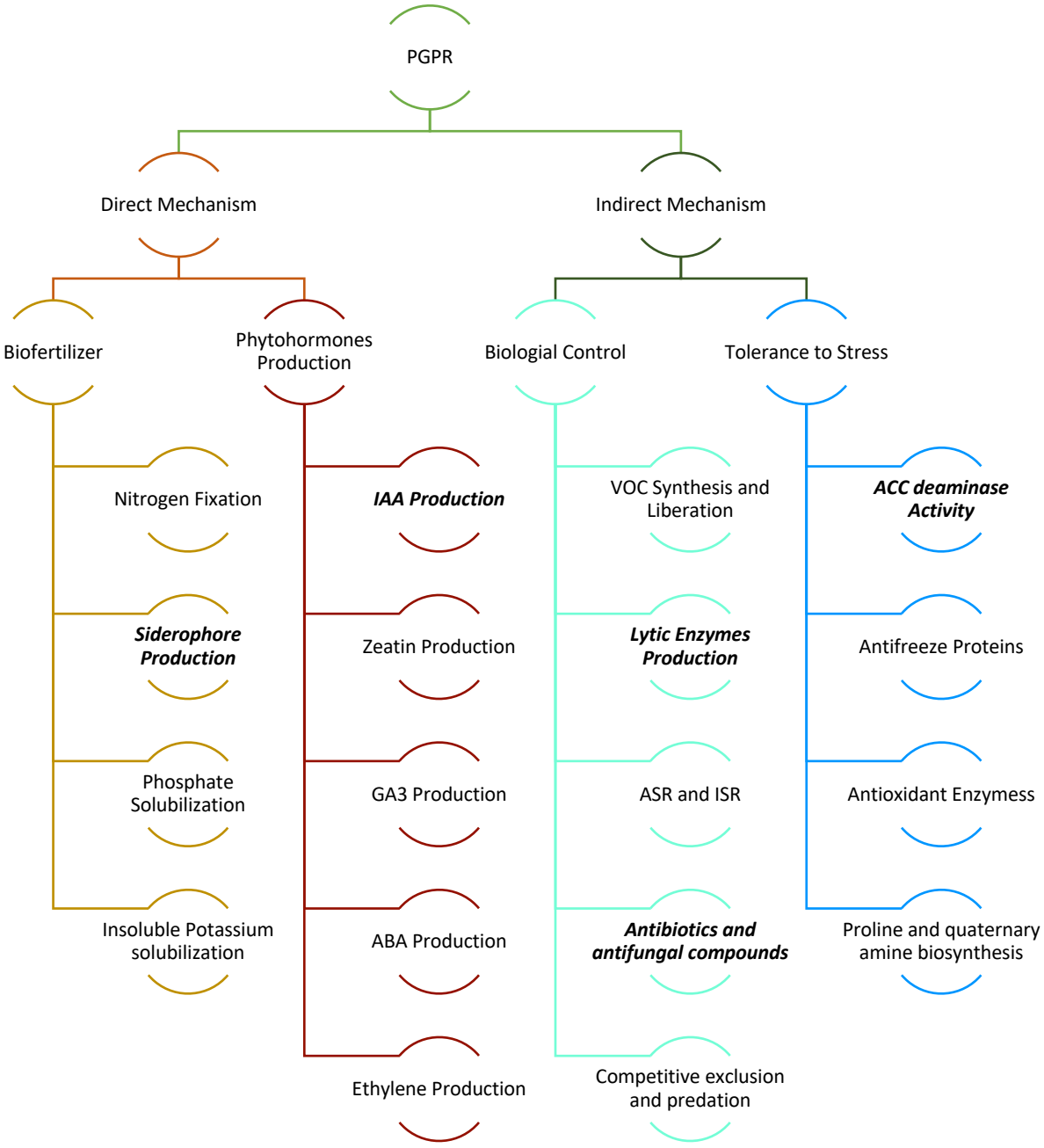

Figure 2.6.6. Mechanisms employed by plant growth promotion bacteria in plant protection (Figueiredo et al., 2016).

Chapter 3

3. Identification and Biochemical Characterization of Culturable Bacterial Endophytes Associated with Cacao Seeds.

3.1. Biological Material

Peruvian organic fermented cacao seeds belonging to the variety CCN-51 were commercially obtained from the Yupik™ store located in Montreal Canada (1385 Louvain Ouest, Montreal, Quebec). The cacao variety CCN-51, was generated in 1965 by the agronomist Homero Castro Zurita in Ecuador. CCN refers to the Collection Castro Naranjal and its numbering 51 refers to the number of crosses made to obtain the desired variety (Ministerio de Agricultura y Riego de Perú, 2016). The seed's flavor is weak with a thin fruity overlay, acidic pulp, and high cocoa-butter content due to abundant polyphenols (Rottiers et al., 2019). This variety is known for its high productivity per hectare, and It is moderately resistant to diseases such as witch's broom, frosty pod rot, and brown rot **(Figure 3.8.1)**, (Turnbull & Hadley, 2020).

3.2. Surface Sterilization and Recovery of microbes Using Culture Dependent Methods

The following protocol was performed four times. At each time, four seeds of the CCN-51variety were surface sterilized using a solution of 2% of sodium hypochlorite for 2 minutes followed by two rinses in sterile distilled water. The effectiveness of the sterilization procedure was tested using the imprint method (Schulz et al., 1993). An absence of growth on the culture medium indicated the surface sterilization method was effective. Under aseptic conditions, the hard seeds were mechanically crushed in a blender into small pieces followed by grinding the pieces using a mortar and a pestle to obtain a homogeneous paste. One gram of the sample was serially diluted (10^{-1}–10^{-5} mL). An aliquot of 100 µL from each dilution was plated in duplicates on the following culture media: Potato Dextrose Agar (PDA), DeMan-Rogosa Sharpe Agar

(MRS Agar) Nutrient Agar (NA), Tryptic Soy Agar (TSA) Malt Extract Agar (MEA) and Luria Bertani Agar (LBA), **(Appendix I)**. All plates were incubated at 30ºC. Single-cell colonies were picked and passed through three rounds of single colony isolation. For long-term storage, single-cell bacterial colonies were stored in glycerol 30% at -80°C (Gagné-Bourgue et al., 2013).

3.3. DNA Extraction and Molecular Identification

Single-cell colonies were grown in 3 mL of liquid media (LB broth) at 30°C for 24 hours with constant agitation (200 rpm) or until the strains reach 1 at OD_{600}. The culture was pelleted by centrifugation at the highest speed for 10 minutes, and the pellet was washed twice with sterile distilled water to remove any remaining culture medium.

Genomic DNA was extracted using the direct colony PCR modified method of Dashti and collaborators (2009). Briefly, the pellet was resuspended in 200 µL of sterile distilled water, the samples were incubated at 98°C for 10 min in a T100™ Bio-Rad Thermal cycler (USA) and immediately after, they were submerged in liquid nitrogen for 10 min. The samples were centrifuged for 10 min and the supernatant was collected. DNA quality was visualized on a 1% agarose gel before subsequent amplification. Likewise, DNA concentration and purity were confirmed spectrophotometrically with a NanoDrop ND1000 spectrophotometer (NanoDrop, Wilmington, DE, United States).

The 16S rDNA fragments regions V1 to V3 **(Figure 3.8.2)** were amplified using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5′-ATTACCGCGGCTGCTGGCA-3′). The PCR reactions were prepared for each sample using 2 µL of DNA, 0.5 µL of each primer at a concentration of 10 µM, 12.5 µL of 2X PCR Taq MasterMix, and 9.5 µL of MiliQ water. Amplifications were carried out on a T100™ Bio-Rad Thermal cycler (USA) with 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1minute. There was a final 5 minutes extension step at 72°C. Amplified products were purified and sequenced at Genome Quebec (Montreal, QC, Canada). Sequences were assessed using the NCBI BLAST tool

(https://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates were identified based on the lowest expected (E) value considered to be the most significant match (Gagné-Bourgue et al., 2013; Scott et al., 2018).

3.4. Phylogenetic Tree

All the analyses were performed using the software Geneious Prime® 2020.1.1. Reverse sequences were obtained from the bacterial DNA amplified using the primer 534R. The sequences were aligned using the CLUSTAL Omega tool. The phylogenetic tree was generated using Neighbor-Joining and used a Subtree-Pruning-2.5. Positions with fewer than 95% site coverage were eliminated. The bootstrap consensus tree was inferred from 1000 replicates, and only branches above a 50% bootstrap score were displayed. The analysis for bacteria involved 466 nucleotide sequences. All positions containing gaps and missing data were eliminated.

3.5. Biochemical Tests

Classically, plant endophytic organisms are grouped concerning to their functional role: plant colonizers, growth promoters, and antagonistic agents **(Section 2.5)**. To screen for these functions, they must synthesize key molecules that ensure success in the performance of a certain task. Researchers have published protocols to evaluate the potential role of the microorganisms quantitatively or qualitatively applying biochemical tests, **(Appendix II)**. The following tests were performed on all 53 bacterial morphotypes isolated from cacao seeds.

3.5.1. Plant Colonization Traits

These tests assess qualitatively the biochemical response generated by the bacteria to a given substrate such as amylases, alginate-lyasess, cellulase, and proteases **(Appendix III)**. A single colony from each isolate was streaked in the center of a Petri plate to evaluate the size of the halo (i.e., the clearing zone) around the colony. In all cases, a clearing zone $\geq$1 mm and extending from the periphery of the spot growth was considered positive (Gagné-Bourgue et al., 2013). The plates were incubated for 48 hours at 28 ºC. The tests for the production of amylases, alginate

lyases, and cellulases but not proteases are Lugol- based, and require this solution to visualize the result (Saleh et al., 2019). After 48 hours, the Petri plates were flooded with Lugol solution for two minutes. In the case of the protease test the plates were incubated for one week, this test does not require Lugol to be revealed.

3.5.2. Plant Growth Promotion

- *ACC (1-aminocyclopropanecarboxylic acid) Deaminase Test*

The inhibition of ethylene is a well-known mechanism of plant growth promotion induced by fungi and bacteria (Matilla & Krell, 2018). Some microorganisms can produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the immediate precursor of the plant hormone ethylene, to produce α-ketobutyrate and ammonia (Glick, 2014). The enzyme ACC deaminase, ensures that the ethylene level does not become elevated which helps to the formation of longer roots and enhances the survival of seedlings, especially during the first few days after the seeds are planted.

Bacterial strains were grown on 5 mL of LB media at 30ºC with constant shaking (200 rpm). The adjustment of cell density was based on standard curves at an absorbance of 600 nm (A600) to plate counts on LB agar (LBA) plates. Cells were pelleted by centrifugation at 8000 g for 10 minutes at 4ºC. The pellets were washed with DF Salts minimal medium (Medium1, **Appendix IV**) and suspended in DF Salts minimal medium (Medium 2 **Appendix IV**) amended with a 0.5 M ACC solution. The samples were incubated for 24 hours at 30ºC with constant shaking (200 rpm). The cells were harvested by centrifugation as previously described and washed two times by suspending the pellet in 0.1 M Tris-HCl pH 7.6, and in 0.1 M Tris-HCl, pH 8.5 respectively. The cells were then toluenized and mixed with 0.5 M of ACC, the suspension was incubated at 30ºC for 15 minutes, centrifuged, and suspended in 0.56 M HCl plus 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 M HCl). The suspensions were vortexed and then incubated at 30ºC for 30 minutes, adding 2 M NaOH. The absorbance was measured at 540 nm (Penrose and Glick, 2003).

- *Calcium solubilization*

Due to climate change, soil conditions have been changing dramatically, affecting the survival of crops in areas where before they were produced. Some bacteria can sequester calcium ions, reducing soil salinity, and improving water uptake by the plant (Shrivastava and Kumar, 2015).

Bacterial strains were assessed for calcium solubilization by streaking the strains on culture media amended with the following components: Yeast: 0.2% CaCo3: 0.2% Glucose: 2 % Agar: 1.5% in 1 L of distilled water. Hydrolysis of calcite is indicated by a clear halo around the bacterial culture (Saleh et al., 2019).

- *Production of Organic Acids*

Organic acids like citric, acetic, tartaric, oxalic, lactic, and malic are metabolites able to dissolve minerals and release nutrients like potassium, aluminium, and silicon to the rhizosphere make them available to the plant (Kumar et al., 2012).

Bacterial strains were grown on 5 mL of Glucose Phosphate Broth media (peptone 7g, dextrose 5 g, K_2HPO_4 5 g in 1 L of distillated water. pH 6.9 $\pm$ 2) at 30ºC during 4 days with constant shaking (200 rpm), 5 drops of methyl red solution were added to the cultures (50 g methyl red dissolved in 1.86 mL of 0.1 M NaOH and 50 mL ethanol, and 100 mL of distilled water). Methyl Red is a pH indicator that changes color in the presence of acidic solutions. Red in pH <4.4, was scored as positively deep orange as a weak reaction and yellow in pH >6.2, as negative. Pure LB and HCl were used as negative and positive controls, respectively (Scott et al., 2018).

- *Production of indole 3-acetic acid (IAA)*

The release of auxin indole-3-acetic acid (IAA) by plant-associated bacteria enhances plant growth and development by improving the root architecture. It increases the root growth as well as root length, along with proliferation and elongation of root hairs (Dubey et al., 2017).

The production of IAA by bacterial strains was performed using the colorimetric assay described by Gordon & Weber, (1950). Bacterial strains were grown in 125 mL flasks with 5 mL of LB medium amended with L-tryptophan 0.1% (IAA precursor) for 5

days at 30ºC, with constant shaking (200 rpm). The samples were centrifuged at maximum speed for 10 minutes, 1 mL of the supernatant was taken and mixed with 2 mL of the Salkowski Reagent (2 mL 0.5 M $FeCl_3$, 49 mL 70% Perchloric Acid, 49 mL distilled water), this mix was incubated at room temperature in the dark for 25 minutes. The absorbance of the samples was measured at 530nm (Gordon & Weber, 1950). This test was performed twice on all 53 isolates, and on selected strains, this test was repeated three times.

- *Siderophore Production, Chrome-Azurol S (CAS) assay*

Siderophores are small, high-affinity iron-chelating compounds that are secreted by microorganisms and serve primarily to transport iron across cell membranes. Iron is one of the microelements less available for the plant in the soil (Amy & Haldeman, 2018).

In order to evaluate the siderophore production by endophytic bacteria, a single colony was streaked in the center of the CAS-agar components are: 100 mM PIPES, 18 mM NH_4Cl, 22 mM KH_2PO_4, 2% (wt/vol) NaCl, 0.3% casamino acids, 0.2% (wt/vol) glucose, 10 µM $FeCl_3$, 58 µM CAS, and 80 µM HDTMA (Louden et al., 2011). The development of a yellow halo formed around the colony indicates a positive siderophore production.

3.5.3. Test for Antimicrobial Properties

- *Sensitivity to Antibiotics*

The bacteria strains isolated were streaked them on LB solid media amended with filter-sterilized antibiotics **(Appendix V)** in the following concentrations: 100 $\mu L/mL^{-1}$ of kanamycin, rifampicin (Sigma-Aldrich), streptomycin (Bioshop, ON, Canada), and with 125 $\mu L/mL^{-1}$ of tetracycline (Fisher Scientific) ampicillin, and chloramphenicol (ICN Biomedicals, Cleveland, OH, USA). Bacteria were considered sensitive to an antibiotic at the concentration tested if no visible growth was after 48 hours of incubation at 28°C (Gagné-Bourgue et al., 2013).

- *Colloidal Chitin Test*

Some bacteria produce lytic enzymes able to degrade chitin, which is a structural polysaccharide of the cell walls founded in species such as fungi and insects (Veliz et al., 2017). The production of chitinases was performed using colloidal chitin (Sigma-Aldrich). The culture medium composition is the following for preparing a liter: colloidal chitin 15 g, peptone 5 g, yeast 0.25 g, glucose 1 g, and agar 15 g in 1 L of distilled water at pH 6.8–7.2. One single bacteria colony was streaked on the center of the plate and incubated at 28°C for 1 week. Clearing zones produced around the colony on the media indicate the production of chitinases, a few drops of Bromothymol blue dye can be added to intensify the contrast. (Murthy & Bleakley, 2012).

- *Hydrogen cyanide (HCN) Production Qualitative Assay*

The HCN is a volatile metabolite that can inhibit the growth of plant pathogens and thereby suppress diseases (Castellano-Hinojosa & Bedmar, 2017).

One single bacteria colony was streaked on solid LBA medium amended with glycine at 4.4 g/L^{-1} (added in solution and filter sterilized). Whatman sterile filter paper grade 1 statured with a picric acid solution (Picric acid 0.5%; 12.5 g of Na_2CO_3, 1 L of distilled water) was placed on the lid of the Petri Dish. The plates were sealed with parafilm and incubated at 28ºC for 48 hours. A change in coloration from yellow to light orange or discoloration was recorded as a positive reaction (Reetha et al., 2014).

3.6. Results

3.6.1. Diversity and abundance of isolated bacteria

Six different microbiological culture media were used to ensure the isolation of a large number of morphotypes. The following culture media were used: Nutrient Agar (NA), Tryptic Soy Agar (TSA) Malt Extract Agar (MEA), and Luria Bertani Agar (LBA), **(Appendix I)**. There was an absence of bacterial growth on Potato Dextrose Agar and DeMan, Rogosa Sharpe Agar media. After successive rounds of isolation, 53 morphotypes were putatively identified via partial sequencing **(Table 3.7.1)**. The majority (60%) of the isolated morphotypes belonged to the genus *Bacillus* (32 isolates), followed by 17% *Enterobacter* (9 isolates), 13%

Klebsiella (7 isolates), 6% *Enterococcus* (3 isolates), 2% *Paneobacillus* (1 isolate) and 2% *Staphylococcus* (1 isolate), **(Figure 3.8.3)**.

The *Bacillus* genus is the most diverse, with 12 different species **(Table 3.7.1)**. The four most abundant species are *Bacillus megaterium*, *B. altitudinis, B. clausii,* and *B. nealsonii* **(Figure 3.8.4)**.

The phylogeny of *Bacillus* species described the **Figure 3.8.5** reveals that all the strains fit into three different evolutionary clusters, the first group is comprised of members of *B. megaterium and B. aryabhattai*, the second group is formed of *B. circulans, B, cytotoxicus, B. kochii and B. nanhaiensis* and third is composed of group *B. xiamenensis and B. altitudinis.*

3.6.2. Biochemical characterization of the isolates

Among the plant colonization traits assessed, four tests were performed to screen the ability of the bacteria to produce specific lytic enzymes. Sixty 60% (32 isolates) can produce amylases, followed by 50% (27 isolates) that produce cellulases and 45% (24 Isolates) can produce alginate. Strains of *B. megaterium* and *B. aryabhattai* were able to produce all of the four enzymes required for colonization followed by more than one *Paenibacillus illinoisensis, Staphylococcus warneri* that were able to produce three out of four enzymes **(Figure 3.8.6 A; Table 3.7.2).**

To evaluate the bacterial isolates for resistance and/or susceptibility to antibiotics, all 53 isolates were tested on media LBA media amended with six different antibiotics **(Appendix V)** Among the 53 morphotypes, 39% (21 isolates) are resistant to ampicillin, while few isolates displayed resistance to rifampicin 8% (4 isolates), tetracycline 7% (3 isolates), and streptomycin 2% (1) in some cases one strain was resistant to more than one antibiotic (i.e. *Enterobacter, E. tabaci, K. variicola*), **(Figure 3.8.6 B; Table 3.7.3)**.

The 53 bacterial strains were screened for their ability to produce HCN, siderophores, and chitinases (i.e., antimicrobial traits). The majority exhibited these traits with varying degrees of production. For example, 28 isolates of out of

50 (52%) produced chitinases. While 15 (28%) had the ability to produce siderophores and 7 isolates (13% of isolates) were able to release the chemical compound hydrogen cyanide **(Figure 3.8.6 C; Table 3.7.4)**.

The majority of bacterial isolates 47% (25 isolates) were growth promoters as they had the ability to solubilize calcium, others 37% (20 isolates) produced organic acids **(Figure 3.8.6 D; Table 3.7.4).** All 53 morphotypes were able to produce the phytohormone IAA with varying amounts ranging from 1.3 - 324 mg/ml **(Table 3.7.5)**. Among the highest phytohormone producers are the strains *Klebsiella sp* (strain 52C) followed by *B. aerius* (strain 38C) and *Klebsiella sp* (strain 54C).

In the case of ACC deaminase, all isolates produced negligible amounts **(Table 3.7.5)**.

3.7. Tables

Table 3.7.1. Taxonomic groups isolated from cacao seeds. Putative identification was performed amplifying the gene 16S rRNA. In addition, it is provided their potential role based on scientific reports.

Molecular Identification 16S	Number of Isolates	Similarity% NCBI	Potential Role
Bacillus aerius	1	98.89%	Salt Tolerance
Bacillus altitudinis	4	99%	Biocontrol and Plant Growth-Promoting
Bacillus aryabhattai	2	100%	Production Phytohormones Tolerant Oxidative Stress
Bacillus circulans	1	100%	Plant growth-promoting
Bacillus clausii	3	100%	Biological Control
Bacillus cytotoxicus)	1	100%	No clear
Bacillus kochii	1	100%	Bioremediation Heavy metals
Bacillus megaterium	11	100%	Plant growth-promoting
Bacillus nanhaiensis	1	97.57%	Biocatalytic
Bacillus nealsonii	3	98.93%	Plant growth-promoting
Bacillus subtilis	2	99.34%	Plant growth-promoting
Bacillus xiamenensis	2	96.59%	Plant growth-promoting
Enterobacter	2	99.33%	Plant growth-promoting
Enterobacter asburiae	5	98%	Plant growth-promoting
Enterobacter tabaci	2	98.77%	Plant growth-promoting
Enterococcus casseliflavus	1	96.59%	Plant growth-promoting
Enterococcus sp	2	99.43%	Plant growth-promoting
Klebsiella spp	4	97.63%	Plant growth-promoting
Klebsiella variicola	3	96.77%%	Plant growth-promoting
Paenibacillus illinoisensis)	1	99.30%	Biological Control-Fungi
Staphylococcus warneri	1	100%	Plant growth-promoting

Table 3.7.2. Screening of endophytes associated with cacao seeds for colonization traits

ID	Species	Cellulose	Alginate	Amylase	Protease
38C	*Bacillus aerius*	+	+	-	-
26C	*Bacillus altitudinis*	+++	-	-	+
31C	*Bacillus altitudinis*	+++	-	-	++
33C	*Bacillus altitudinis*	-	-	-	-
9C	*Bacillus aryabhattai*	+++	+++	+++	+++
4C	*Bacillus aryabhattai*	+	+	+++	-
28C	*Bacillus circulans*	-	+++	+++	-
22C	*Bacillus clausii*	-	-	-	-
23C	*Bacillus clausii*	-	-	-	-
24C	*Bacillus clausii*	-	-	-	-
16C	*Bacillus cytotoxicus*	+++	+++	-	-
25C	*Bacillus kochii*	-	-	-	-
10_CL	*Bacillus megaterium*	+++	+	+++	+++
1C	*Bacillus megaterium*	+++	+++	+++	+++
2C	*Bacillus megaterium*	+++	+	+++	+++
3C	*Bacillus megaterium*	+++	+	+++	++
6C	*Bacillus megaterium*	+++	+++	+++	+++
8C	*Bacillus megaterium*	+++	-	+++	+++
10C	*Bacillus megaterium*	+++	+++	+++	+++
5_CL	*Bacillus megaterium*	+	+++	+++	+++

ID	Species	Cellulose	Alginate	Amylase	Protease
14CL	*Bacillus megaterium*	+	+++	+++	+++
48C	*Bacillus nealsonii*	+++	-	-	-
15C	*Bacillus subtilis*	-	+++	+++	-
19C	*Bacillus subtilis*	-	+++	+++	+
14C	*Bacillus xiamenensis*	+++	-	-	-
30C	*Bacillus xiamenensis*	-	-	+++	-
42C	*Enterobacter*	++	-	-	-
53C	*Enterobacter*	++	-	-	-
27C	*Enterobacter asburiae*	+++	-	+++	-
36C	*Enterobacter asburiae*	-	++	+++	-
39C	*Enterobacter asburiae*	-	+++	+	-
40C	*Enterobacter asburiae*	+++	-	+++	-
55C	*Enterobacter asburiae*	+++	-	+	-
49C	*Enterobacter tabaci*	+++	-	+++	-
51C	*Enterobacter tabaci*	++	-	-	-
50C	*Enterococcus casseliflavus*	-	-	+	-
29C	*Enterococcus sp.*	-	+++	-	-
32C	*Enterococcus sp.*	-	-	+++	-
37C	*Klebsiella spp*	-	+	+++	-
41C	*Klebsiella spp*	-	+	+++	-
52C	*Klebsiella spp*	-	+	+++	-

ID	Species	Cellulose	Alginate	Amylase	Protease
54C	*Klebsiella spp*	-	-	+++	-
44C	*Klebsiella variicola*	-	+++	-	-
45C	*Klebsiella variicola*	-	-	+	-
47C	*Klebsiella variicola*	++	-	+	-
21C	*Paenibacillus illinoisensis*	+	+++	+++	-
12C	*Staphylococcus warneri*	+	++	+++	-

(-) Negative reaction (+) Positive reaction showing a clearing zone ≤ 0.5cm. (++) Positive reaction showing a clearing zone 0.5cm≤ 1 cm. (+++) Positive reaction showing a clearing zone 1 cm≤ 1.5 cm

Table 3.7.3. Antibiotics resistance tests from all microbes isolated from the cacao seeds.

ID	Genus and Species	Ampicillin	Chloramphenicol	Kanamycin	Rifampicin	Streptomycin	Tetracycline
38C	*Bacillus aerius*	+	-	-	-	-	-
26C	*Bacillus altitudinis*	-	-	-	-	-	-
31C	*Bacillus altitudinis*	-	-	-	-	-	+
33C	*Bacillus altitudinis*	-	-	-	-	-	-
9C	*Bacillus aryabhattai*	-	-	-	-	-	-
4C	*Bacillus aryabhattai*	+	-	-	-	-	-
28C	*Bacillus circulans*	-	-	-	-	-	-
22C	*Bacillus clausii*	-	-	-	-	-	-
23C	*Bacillus clausii*	-	-	-	-	-	-
24C	*Bacillus clausii*	-	-	-	-	-	-
16C	*Bacillus cytotoxicus*	-	-	-	-	-	-
25C	*Bacillus kochii*	+	-	-	-	-	-
10_CL	*Bacillus megaterium*	-	-	-	-	-	-
1C	*Bacillus megaterium*	-	-	-	-	-	-
2C	*Bacillus megaterium*	-	-	-	-	-	-
3C	*Bacillus megaterium*	+	-	-	-	-	-
6C	*Bacillus megaterium*	+	-	-	-	-	-
8C	*Bacillus megaterium*	+	-	-	-	-	-
10C	*Bacillus megaterium*	-	-	-	+	-	-
5_CL	*Bacillus megaterium*	-	-	-	-	-	-
14CL	*Bacillus megaterium*	+	-	-	-	-	-
48C	*Bacillus nealsonii*	-	-	-	-	-	-
15C	*Bacillus subtilis*	-	-	-	-	-	-
19C	*Bacillus subtilis*	-	-	-	-	-	-

ID	Genus and Species	Ampicillin	Chloramphenicol	Kanamycin	Rifampicin	Streptomycin	Tetracycline
14C	*Bacillus xiamenensis*	-	-	-	-	-	-
30C	*Bacillus xiamenensis*	-	-	-	-	-	-
42C	*Enterobacter*	+	-	-	+	-	-
53C	*Enterobacter*	+	-	-	-	-	-
27C	*Enterobacter asburiae*	+	-	-	-	-	-
36C	*Enterobacter asburiae*	-	-	-	-	-	-
39C	*Enterobacter asburiae*	-	-	-	-	-	-
40C	*Enterobacter asburiae*	+	-	-	-	-	-
55C	*Enterobacter asburiae*	+	-	-		-	-
49C	*Enterobacter tabaci*	+	-	-	-	-	+
51C	*Enterobacter tabaci*	+	-	-	+	-	-
50C	*Enterococcus casseliflavus*	-	-	-	-	-	-
29C	*Enterococcus sp.*	-	-	-	-	-	-
32C	*Enterococcus sp.*	-	-	-	-	-	-
37C	*Klebsiella spp*	+	-	-	-	-	-
41C	*Klebsiella spp*	+	-	-	-	-	-
52C	*Klebsiella spp*	+	-	-	-	-	-
54C	*Klebsiella spp*	+	-	-	-	-	-
44C	*Klebsiella variicola*	+	-	-	-	-	+
45C	*Klebsiella variicola*	+	-	-	-	-	-
47C	*Klebsiella variicola*	+	-	-	+	-	-

ID	Genus and Species	Ampicillin	Chloramphenicol	Kanamycin	Rifampicin	Streptomycin	Tetracycline
21C	*Paenibacillus illinoisensis*	-	-	-	-	+	-
12C	*Staphylococcus warneri*	-	-	-	-	-	-

(-) No growth (+) Growth

Table 3.7.4. Antimicrobials traits of bacterial endophytes isolated from cacao seeds.

ID	Genus	Chitinase*	Calcium Solubilization	HCN**	Organic Acid**	Siderophore***
38C	*Bacillus aerius*	++	++	-	+	+
26C	*Bacillus altitudinis*	+	-	-	-	-
31C	*Bacillus altitudinis*	+	-	-	++	-
33C	*Bacillus altitudinis*	-	+	-	-	-
9C	*Bacillus aryabhattai*	+	-	-	-	-
4C	*Bacillus aryabhattai*	++	-	-	-	-
28C	*Bacillus circulans*	-	-	-	++	-
22C	*Bacillus clausii*	-	-	-	+	-
23C	*Bacillus clausii*	-	-	-	+	-
24C	*Bacillus clausii*	-	-	-	+	-
16C	*Bacillus cytotoxicus*	+	-	-	+	-
25C	*Bacillus kochii*	+++	-	-	+	-
10_CL	*Bacillus megaterium*	++	+	-	-	-
1C	*Bacillus megaterium*	+	+	-	+	-
2C	*Bacillus megaterium*	+	+	-	-	-
3C	*Bacillus megaterium*	+	+	-	-	-
6C	*Bacillus megaterium*	+	-	-	-	-
8C	*Bacillus megaterium*	++	-	-	+	-
10C	*Bacillus megaterium*	+	+	-	-	-
5_CL	*Bacillus megaterium*	+	+	-	+	-
14CL	*Bacillus megaterium*	+	-	-	-	-

ID	Genus	Chitinase*	Calcium Solubilization	HCN**	Organic Acid**	Siderophore***
48C	*Bacillus nealsonii*	++	-	-	++	-
15C	*Bacillus subtilis*	++	-	-	+	-
19C	*Bacillus subtilis*	+	-	-	+	-
14C	*Bacillus xiamenensis*	+++	-	-	-	-
30C	*Bacillus xiamenensis*	-	-	-	+	-
42C	*Enterobacter*	+	++	-	-	++
53C	*Enterobacter*	+	+	+	-	+
27C	*Enterobacter asburiae*	-	+	-	-	++
36C	*Enterobacter asburiae*	-	-	-	-	-
39C	*Enterobacter asburiae*	-	+	-	++	-
40C	*Enterobacter asburiae*	+	+	-	-	++
55C	*Enterobacter asburiae*	-	+	-	-	++
49C	*Enterobacter tabaci*	+	+	+	-	++
51C	*Enterobacter tabaci*	+	++	-	-	++
50C	*Enterococcus casseliflavus*	-	-	-	++	-
29C	*Enterococcus sp.*	-	++	-	++	-
32C	*Enterococcus sp.*	-	-	-	-	-
37C	*Klebsiella spp*	-	++	-	-	++
41C	*Klebsiella spp*	-	+	+	-	+
52C	*Klebsiella spp*	+++	+	-	+	+
54C	*Klebsiella spp*	-	++	+	-	+
44C	*Klebsiella variicola*	+++	+	+	-	+
45C	*Klebsiella variicola*	+++	+	+	-	+

ID	Genus	Chitinase*	Calcium Solubilization	HCN♣	Organic Acid**	Siderophore***
47C	*Klebsiella variicola*	-	++	+	-	+
21C	*Paenibacillus illinoisensis*	-	-	-	++	-
12C	*Staphylococcus warneri*	-	+	-	++	-

(-) Negative reaction *(+) Positive reaction showing a clearing zone ≤ 0.5cm. (++) Positive reaction showing a clearing zone 0.5cm≤ 1 cm. (+++) Positive reaction showing a clearing zone 1 cm≤ 1.5 cm

**Deep red was scored as positive (++), deep orange was scored as a weak reaction (+)

***(+) Positive reaction showing growth ≤ 0.5cm. (++) Positive reaction showing growth 0.5cm≤ 1 cm. (+++) Positive reaction showing growth 1 cm≤ 1.5 cm

♣ Decolouration of the filter paper was considered as positive.

Table 3.7.5. IAA and ACC deaminase production of endophytes associated with seeds.

Internal Code	ID	IAA μg/ml	ACC deaminase activity (nmol α-Ketobutyrate mg^{-1} $30min^{-1}$) [a] ±SE
38C	*Bacillus aerius*	307.7±23.6	0.08±0.00
26C	*Bacillus altitudinis*	34.9±1.9	0.03±0.00
31C	*Bacillus altitudinis*	33.2±2.4	0.01±0.00
33C	*Bacillus altitudinis*	11.4±0.5	0.03±0.00
9C	*Bacillus aryabhattai*	148.5±1.3	0.06±0.00
28C	*Bacillus aryabhattai*	22.3±0.2	0.01±0.00
22C	*Bacillus circulans*	6.0±0.2	0.00±0.00
23C	*Bacillus clausii*	13.4±1.3	0.02±0.01
24C	*Bacillus clausii*	9.0±3.3	0.01±0.00
16C	*Bacillus clausii*	0.0±0.1	0.07±0.00
25C	*Bacillus cytotoxicus*	41.5±0.5	0.00±0.00
1C	*Bacillus kochii*	109.7±1.5	0.04±0.01
2C	*Bacillus megaterium*	125.4±0.6	0.03±0.01
3C	*Bacillus megaterium*	122.6±3.2	0.03±0.00
4C	*Bacillus megaterium*	124.6±3.0	0.01±0.00
6C	*Bacillus megaterium*	145.7±3.0	0.04±0.00
8C	*Bacillus megaterium*	94.9±2.9	0.03±0.00
10C	*Bacillus megaterium*	126.0±2.0	0.01±0.00
5_CL	*Bacillus megaterium*	139.1±2.9	0.04±0.00
10_CL	*Bacillus megaterium*	1.3±1.4	0.03±0.00
14CL	*Bacillus megaterium*	141.7±6.4	0.09±0.00
48C	*Bacillus nealsonii*	26.4±1.7	0.07±0.00
19C	*Bacillus. subtilis*	30.7±1.4	0.02±0.00
14C	*Bacillus. xiamenensis*	15.9±2.4	0.03±0.00
15C	*Bacillus subtilis*	46.7±0.2	0.03±0.00
39C	*Enterobacter asburiae*	3.4±2.6	0.02±0.00
27C	*Enterobacter asburiae*	253.2±32.8	0.09±0.00
36C	*Enterobacter asburiae*	29.3±0.7	0.01±0.00
40C	*Enterobacter asburiae*	264.0±55.8	0.09±0.00
53C	*Enterobacter sp*	307.1±24.4	0.00±0.00
42C	*Enterobacter sp.*	181.6±5.7	0.05±0.00
49C	*Enterobacter tabaci*	278.9±15.2	0.03±0.00
51C	*Enterobacter tabaci*	276.7±5.2	0.07±0.01

Internal Code	ID	IAA μg/ml	ACC deaminase activity (nmol α-Ketobutyrate mg^{-1} $30min^{-1}$) [a] ±SE
50C	*Enterococcus casseliflavus*	11.7±2.1	0.02±0.00
29C	*Enterococcus sp.*	5.9±0.6	0.02±0.00
32C	*Enterococcus sp.*	22.3±2.6	0.04±0.00
45C	*Klebsiella variicola*	299.4±35.4	0.06±0.00
47C	*Klebsiella variicola*	219.7±54.7	0.02±0.00
44C	*Klebsiella variicola*	324.4±0.0	0.00±0.00
37C	*Klebsiella spp*	189.8±23.2	0.04±0.00
41C	*Klebsiella spp*	290.9±47.4	0.06±0.00
52C	*Klebsiella spp*	324.4±0.0	0.08±0.00
54C	*Klebsiella spp*	307.0±24.5	0.05±0.00
21C	*Paenibacillus illinoisensis*	0.0±0.1	0.01±0.00
12C	*Staphylococcus warneri*	12.8±1.1	0.02±0.00

Table 3.7.6. Selected strains with exceptional biochemical traits, mostly based on the production of IAA and siderophore.

Internal Code	ID	Gram Stain	IAA μg/ml	HCN♣	Siderophore***
38C	*Bacillus aerius*	+	307.72 ± 23.6	-	+
49C	*Enterobacter tabaci*	-	278.93 ± 15.2	+	++
53C	*Enterobacter spp*	-	307.11 ± 24.4	+	+
51C	*Enterobacter tabaci*	-	276.70 ± 5.2	-	++
44C	*Klebsiella varicola*	-	324.38 ± 0.0	+	+
52C	*Klebsiella spp*	-	324.38 ± 0.0	+	+

♣ Decolouration of the filter paper was considered as positive

***(+) Positive reaction showing growth ≤ 0.5cm. (++) Positive reaction showing growth 0.5cm≤ 1 cm. (+++) Positive reaction showing growth 1 cm≤ 1.5 cm

3.8. Figures

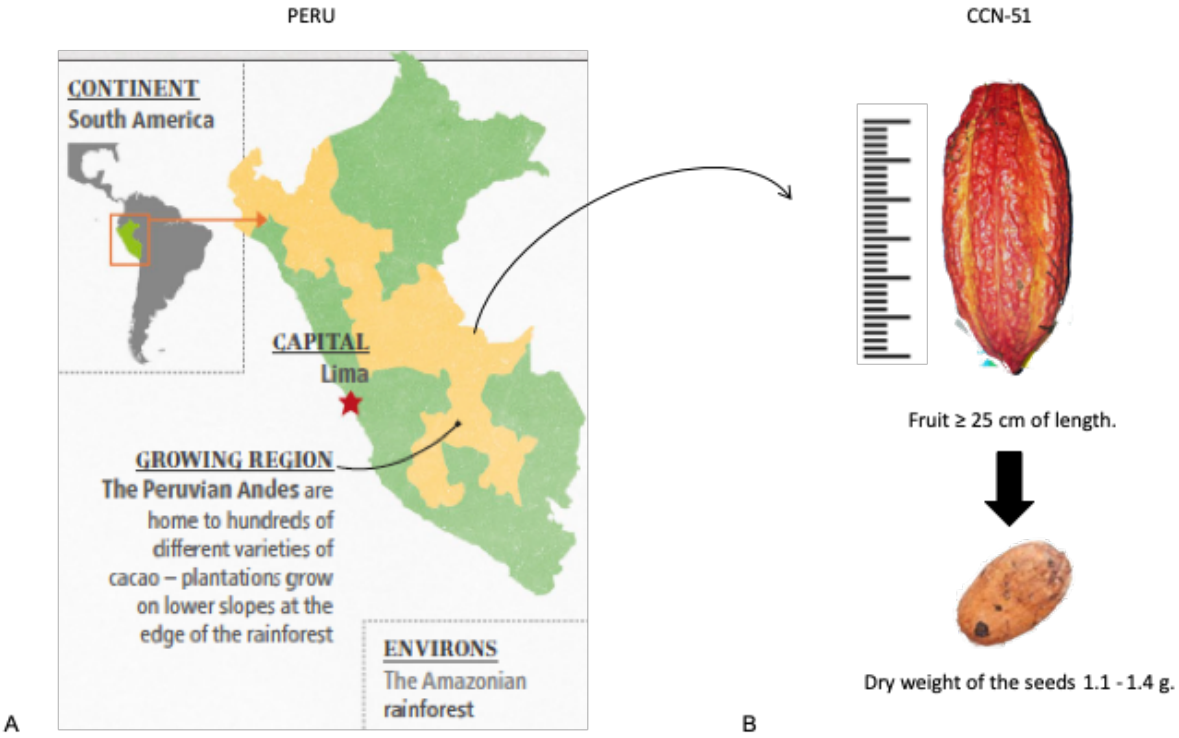

Figure 3.8.1. A. Map of Peru showing the productive areas of cacao (Ramsey, 2016). **B.** Pod and seed of the cacao variety CCN-51(Turnbull & Hadley, 2020)

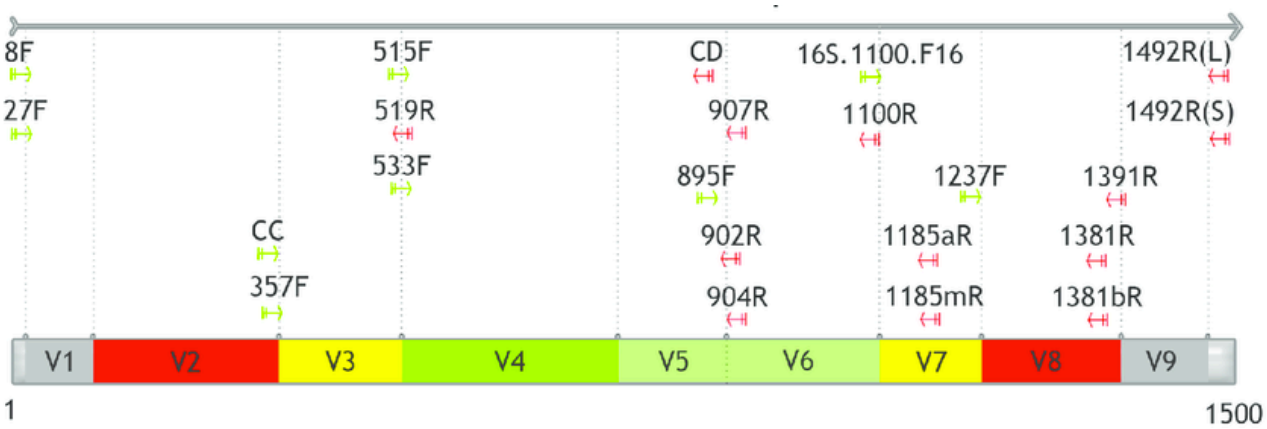

Figure 3.8.2. Primer Map of the Bacterial Gene 16S rRNA (Yang et al., 2016).

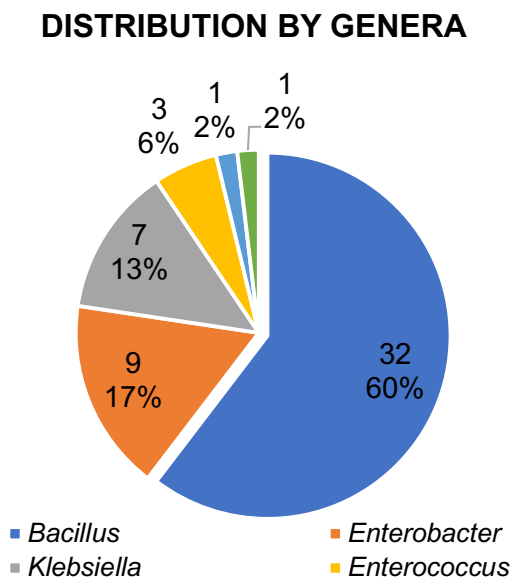

Figure 3.8.3. Diversity and abundance of genera of bacteria isolated from cacao seeds.

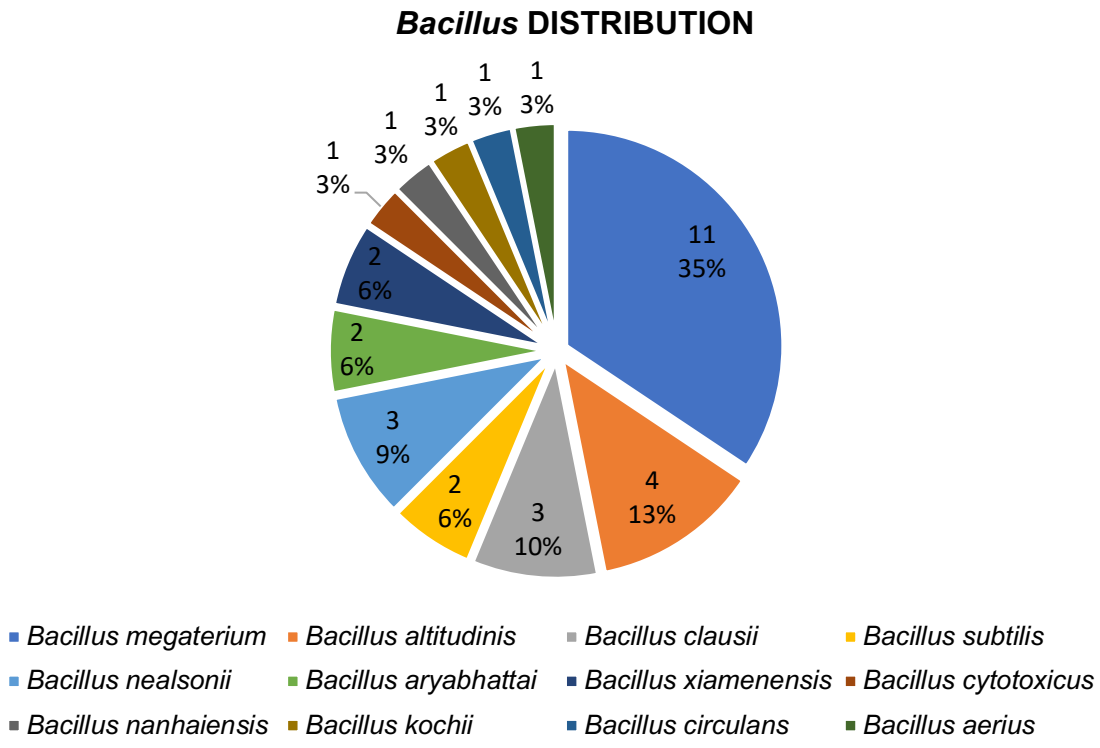

Figure 3.8.4. Diversity and abundance of Bacillus species isolated from cacao seeds.

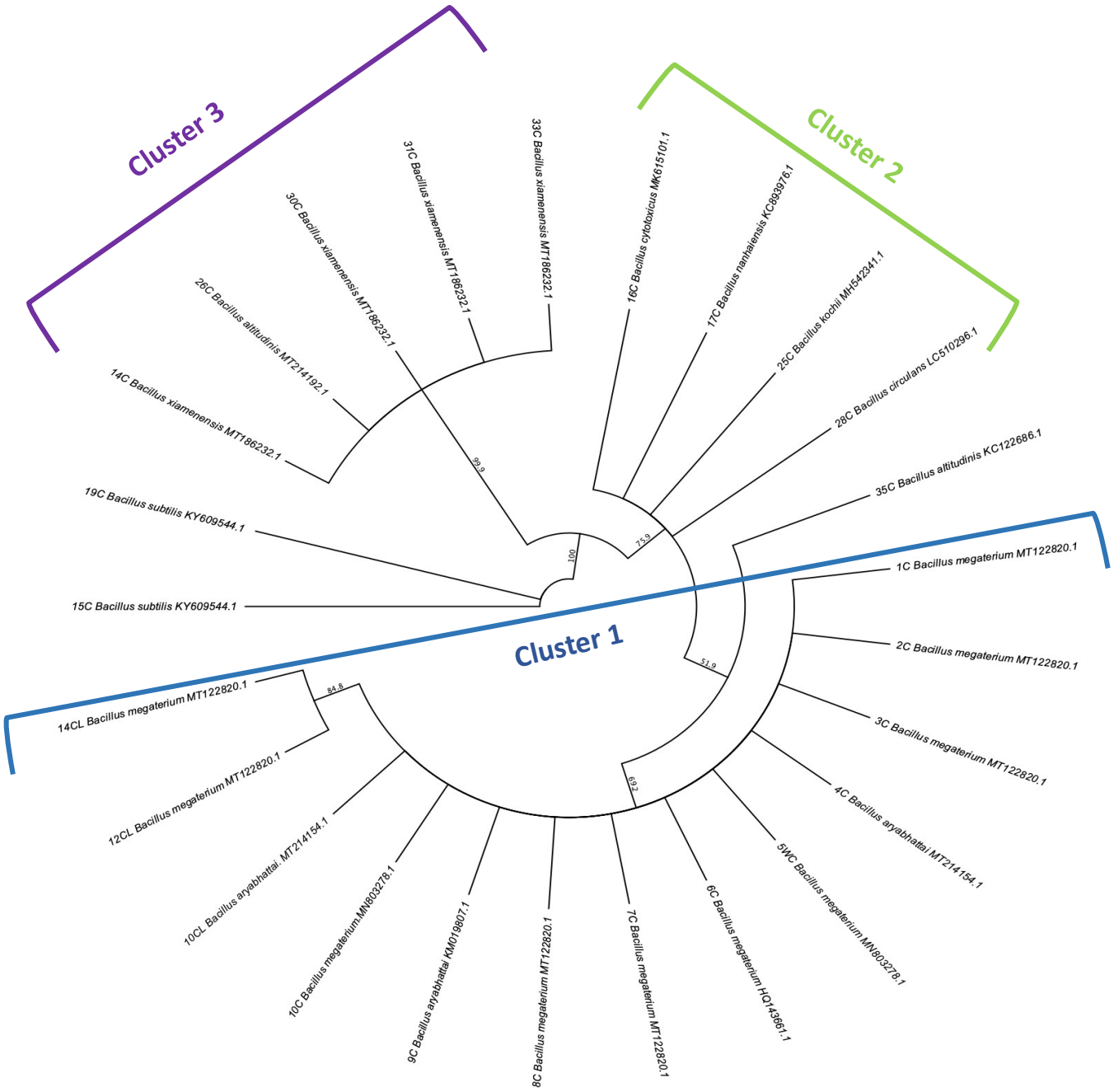

Figure 3.8.5. Neighbor-Joining phylogenetic tree of *Bacillus* species isolated from cacao seeds. The bootstrap consensus tree was inferred from 1000 replicates, and only branches above a 50% bootstrap score were displayed.

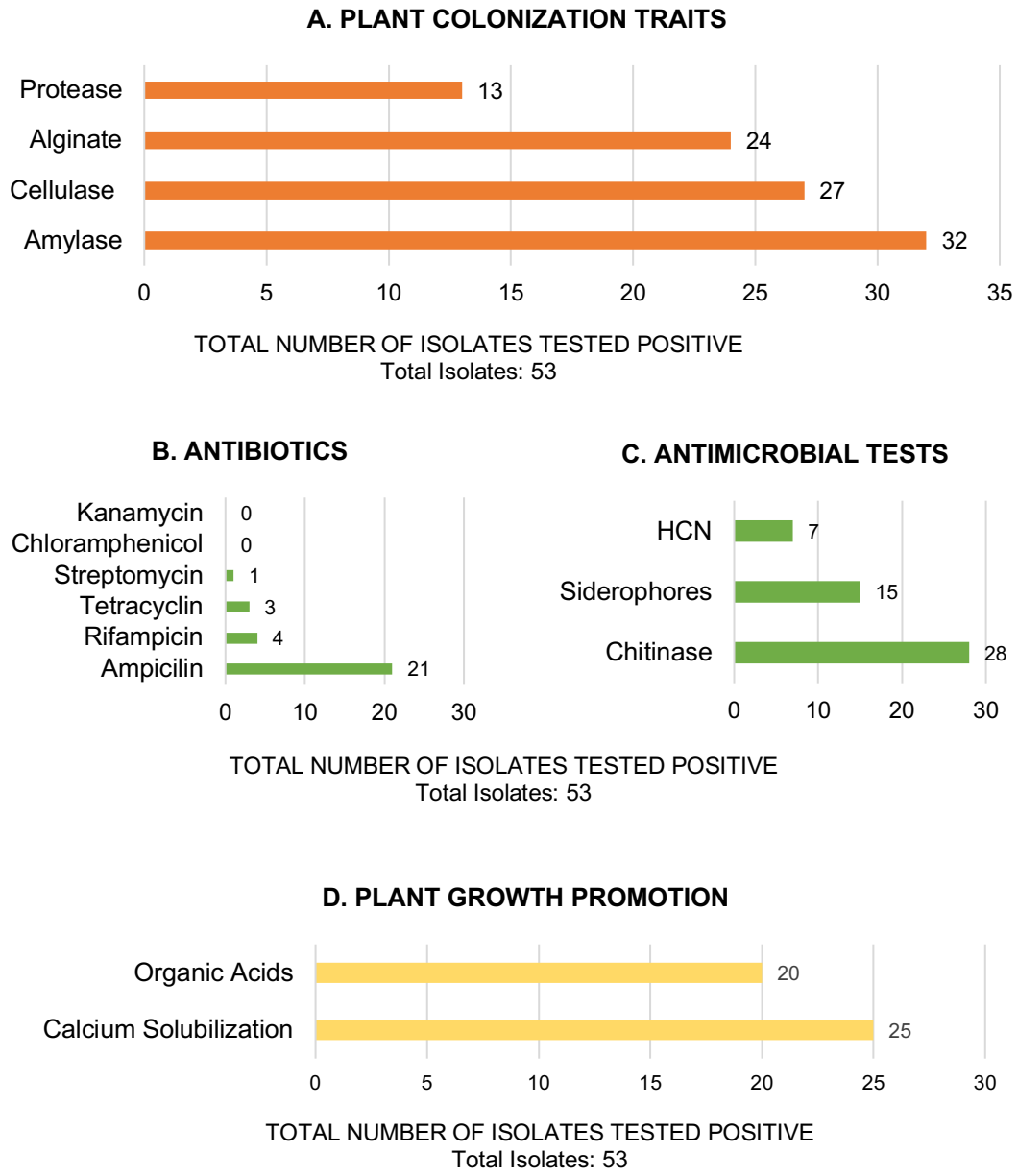

Figure 3.8.6. Number of positive strains from Peruvian cacao seeds tested positive for different biochemical tests. **A.** Bacteria isolates exhibiting to the production produce lytic enzymes. **B**. Resistant to antibiotics. **C.** Bacteria isolates exhibiting the production antifungal and antimicrobial metabolites. **D.** Bacteria isolates exhibiting the production metabolites associated with plant growth promotion.

Chapter 4

4. Identification of Microbes Associated with Susceptible and Tolerant Varieties of Cacao Against Frosty Pod Rot.

During the last ten years, recent findings confirm that the plant microbiome present in the rhizosphere, phyllosphere, and endosphere play important functions related to plant development and survival (Compant et al., 2019). However, the microbial composition of the roots, leaves, stems, and flowers is influenced by biotic and abiotic factors. For example, root microbial communities are shaped by soil conditions (type, moisture, structure, and organic matter) and root exudates. Besides, factors like weather, pathogens, and cultural practices influence the microbiota of the upper and below parts of the plants (Compant et al., 2019).

Bulgarelli et al., (2015) showed that plant genotype can modulate the composition of the microbiome in the rhizosphere and roots, indicating that the mechanisms of defense and root metabolites mainly shaped root microbial community structure. Likewise, factors like plant age and developmental stage, health, and fitness are also known to influence plant bacterial community structure, affecting the exudation profiles and plant signaling (induced systemic resistance, systemic acquired resistance), (Pérez-Jaramillo et al., 2018).

The case of cacao, the use of endophytic microorganisms to control fungal diseases such as *Moniliophthora perniciosa* the causal agent of witches broom was explored by Rubini et al., (2005). A total of 265 endophytic fungi were isolated from leaves, stems, and roots from tolerant, susceptible, and diseased cacao trees. Forty-three of the isolates reduced the growth of *M. perniciosa* under in vitro conditions, and one isolate successfully reduced the disease in the field. Christian et al., (2020) reported that dominant foliar endophytes and cacao pathogens can induce changes in secondary chemical profiles of this tree.

Similarly, tests have been carried out with endospore-forming bacteria from cacao leaves and healthy pods. Of the 69 isolates, eight significantly inhibited *Phytophthora*

capsici in detached leaf assays. This led to increased interest in exploring the bacterial microbiome of cacao trees. Research conducted using isolated cacao's endophytic fungi has shown to be an efficient alternative to synthetic pesticides. However, knowledge on the diversity of bacterial microbiota and their potential uses of the bacteria present in cacao fruits is limited (Melnick et al., 2011).

4.1. Plant Material

Two cacao varieties were selected based on their tolerance to the pathogen *Moniliopthora roreri* were selected. I) The variety CATIE-R6 was released in the year 2007 in Costa Rica by Dr. Wilbert Phillips-Mora (CATIE, 2020). This variety is classified as "Fine or Flavour Cocoa" described as fruity, floral, or nutty due to the presence of secondary metabolites (terpenes, alcohols, esters, aldehydes, and methyl ketones) in the pulp that contributes desirable aroma notes (Hegmann et al., 2017). It is tolerant to *M. roreri* and moderately tolerant to *Phytophthora palmivora* (Turnbull & Hadley, 2020) The CATONGO variety originated from Brazil, where it is commonly known as albino forastero due to the white color of its seed. The albino character of this variety provides a very mild and not incredibly complex flavor. Its aroma consistently has a unique green pepper scent with occasional hints of vanilla (Chocolate Alchemy, 2019). This variety is susceptible to *M. roreri* and is moderately tolerant to *Phytophthora palmivora* (Turnbull & Hadley, 2020)

4.2. Collection sites

The province of Bocas del Toro is known as the cacao producing area in Panama **(Figure 4.7.1)**. Selection and collection of two cacao varieties from La Magnita farm it was done with the collaboration of Mr. Orlando Lozada renowned for the cultivation of high-quality organic cacao, and the owner of La Magnita farm. Two cacao varieties CATIE-R6 and CATONGO were selected from La Magnita Farm (09º22'50.1"N 82º34'27.7"W). From each variety ten (10) healthy mature pods were collected (one fruit per tree). The fruits were labeled and stored individually in Ziploc bags on ice until they reach the Smithsonian Facilities (Panama City).

4.3. Microbial isolation and Culture

The external surface of the pod (exocarp; **Figure 2.6.2**) was sterilized in a solution of 2% sodium hypochlorite. Isolation of microbes associated with the endocarp, pulp, and seed was performed aseptically as depicted in **Figure 2.6.2**.

- Endocarp: From each pod, two opposite sections of 5 cm^2 from the endocarp were excised and cut into pieces of 0.5 cm^2. A total of 40 pieces/10 pods were used for microbial isolation. From each pod, two opposite sections of 5cm^2 from the endocarp were excised and were cut into small pieces of 0.5 cm^2. Per pod, four pieces of 0.5 cm^2 were taken, making a pool with 40 pieces of tissue in total.

- Pulp; The pulp of 10 seeds/10 cacao fruits was sampled and immersed in PBS 1X buffer overnight at room temperature with constant shaking (100 rpm) to dislodge the pulp from the seed. The pulp was then easily removed with the help of a sterile scalpel.

- Seeds; After removing the pulp from the seeds, they were left in buffer PBS 1X one more night, at room temperature with constant shaking (100 rpm) to soften them

Endocarp sections, pulp, and seeds were mechanically homogenized individually followed by grinding the tissue into a paste using a mortar and a pestle as previously described in Chapter 2. A volume of 500 μL of sterile PBS 1X buffer was added to each ground tissue and incubated overnight at room temperature with constantly shaking (100 rpm). An aliquot (100 μL) of each tissue was serially diluted ($1x10^{-1}$-$1x10^{-5}$) of each tissue and plated in duplicates on different microbiological media (**Appendix I)**. All plates were incubated at room temperature (25 + 2°C) and examined regularly for emerging bacterial colonies. Emergent bacteria isolates were picked and passed through three rounds of single-colony isolation for long-term storage, single-cell bacterial colonies were stored glycerol 30% at -80°C (Gagné-Bourgue et al., 2013).

4.4. DNA Extraction and Molecular Identification

DNA extraction from purified bacterial morphotypes was carried out using the same protocol previously described in section 3.3 in Chapter 3.

4.5. Results

4.5.1. Diversity and abundance of isolated bacteria

A total of 160 morphotypes from CATIE-R6 (81) and CATONGO (79), were isolated and cultured from different cacao tissues (seeds, pulp, and endocarp). The highest proportion of bacterial isolates were associated with the seeds of both cultivars (30 and 29 morphotypes respectively) followed by the pulp and endocarp. Interestingly, there was no difference in the abundance of bacterial strains between both varieties **(Figure 4.7.2)**.

Out of 160 isolates, 93 bacterial strains were sequenced and putatively identified including 49 morphotypes from CATIE-R6 and 44 morphotypes from CATONGO varieties **(Tables 4.6.1** and **4.6.2)**.

The diversity of bacterial strains associated with **CATIE-R6** (Tolerant) was mainly associated with the phylum *Proteobacteria*, followed by *Firmicutes* and only a low proportion of strains belonged to the genus *Actinobacteria*. The cacao endocarp had the highest proportion of bacteria genera (70%) followed by the seeds (59%) and the pulp (37%) (**Figure 4.7.3)**.

Some genera are present in more than one layer (**Figure 4.7.4)**. In the variety CATIE-R6, *Acinetobacter* was present in all three tissues. The genera *Pseudomonas* and *Terribacillus* were commonly distributed in the endocarp and the pulp while *Enterobacter, Erwinia, Klebsiella, Kluyvera, Kokasonia,* and *Pantoea* were common in the pulp and the seeds. Interestingly, in the tolerant variety, species of *Brachybacterium, and Tatumella* were tissue-specific only identified in the tolerant variety **(Figure 4.7.7)**.

Similarly, in the **CATONGO** (Susceptible) variety, the proportion of bacteria isolated is distributed among the phylum *Proteobacteria*, *Firmicutes,* and *Actinobacteria.* The distribution of genera in the three tissues varied. The highest proportion of genera was present in the pulp (61%), followed by seeds (26%) and then endocarp (23%) **(Figure 4.7.5)**.

Likewise, in the CATONGO variety, some genera were present in more than one type of tissue **(Figure 4.7.6).** The genus *Terribacillus* was present in the endocarp and the pulp. While *Brevundimonas* and *Enterobacter* were associated with the endocarp and the seeds. However, the pulp and the seeds have in common three genera of bacteria: *Klebsiella, Paenobacillus,* and *Erwinia. Kluverya* and *Pantoea* genera were common to the three tissues. Also, CATONGO had strains associated with three tissues and were not found in the tolerant variety CATIE-R6. These were *Pseudocitrobacter*, *Agrobacterium*, and five different genera isolated from the seeds **(Figure 4.7.7)**.

There were common genera associated with specific tissues of both cacao varieties (CATIE-R6 and CATONGO) **(Figure 4.7.7).** For example, *Terribacillus* and *Pseudomonas* were common in the endocarp of both varieties. The seeds had in common, *Pantoea* and *Kluyvera*. While the pulp of both varieties was associated with *Terribacillus*, *Klebsiella,* and *Erwinia*.

4.6. Tables

Table 4.6.1. List of bacterial species isolated from the cacao variety CATIE-R6 (Tolerant). Putative identification was performed amplifying the gene 16S rRNA. The potential role is provided based of scientific reports.

Layer	Phylum	Molecular Identification 16S	Number of Isolates	Similarity % NCBI
Seeds	*Proteobacteria*	*Acinetobacter sp*	1	99.64%
Seeds	*Firmicutes*	*Bacillus cereus*	1	95.31%
Seeds	*Proteobacteria*	*Enterobacter soli*	1	99.06%
Seeds	*Proteobacteria*	*Enterobacteriaceae bacterium*	2	99.50%
Seeds	*Proteobacteria*	*Erwinia persicina*	1	92.81%
Seeds	*Proteobacteria*	*Klebsiella sp*	1	99.31%
Seeds	*Proteobacteria*	*Klebsiella variicola*	1	98.62%
Seeds	*Proteobacteria*	*Kluyvera ascorbata*	3	99.33%
Seeds	*Proteobacteria*	*Kosakonia cowanii*	2	99.16%
Seeds	*Proteobacteria*	*Pantoea coffeiphila*	1	98.27%
Seeds	*Proteobacteria*	*Pantoea rodasi*	1	99.10%
Seeds	---	*Uncultured bacteria*	1	99.07%
Pulp	*Proteobacteria*	*Acinetobacter oleivorans*	2	98.99%
Pulp	*Proteobacteria*	*Acinetobacter sp.*	1	99.09%
Pulp	*Proteobacteria*	*Enterobacter cloacae*	1	95.79%
Pulp	*Proteobacteria*	*Erwinia persicina*	1	95.86%
Pulp	*Proteobacteria*	*Klebsiella oxytoca*	1	99.08%
Pulp	*Proteobacteria*	*Kluyvera ascorbata*	1	99.83%
Pulp	*Proteobacteria*	*Kosakonia sp*	1	98.94%
Pulp	*Proteobacteria*	*Pantoea agglomerans*	1	99.41%
Pulp	*Proteobacteria*	*Pseudomonas cedrina*	1	98.99%
Pulp	*Proteobacteria*	*Pseudomonas sp*	1	99.12%
Pulp	*Proteobacteria*	*Rosenbergiella australiborealis*	1	98.28%%
Pulp	*Proteobacteria*	*Rosenbergiella sp*	1	99.35%
Pulp	*Proteobacteria*	*Tatumella ptyseos*	2	99.19%
Pulp	*Firmicutes*	*Terribacillus saccharophilus*	3	98.38%
Pulp	*Firmicutes*	*Terribacillus sp*	1	100%
Endocarp	*Proteobacteria*	*Acinetobacter johnsonii*	1	100.00%
Endocarp	*Proteobacteria*	*Acinetobacter sp.*	1	99.67%
Endocarp	*Actinobacteria*	*Brachybacterium conglomeratum*	1	99.56%
Endocarp	*Firmicutes*	*Paenibacillus lautus*	1	98.91%
Endocarp	*Firmicutes*	*Paenibacillus taichungensis*	1	93.60%
Endocarp	*Proteobacteria*	*Pseudomonas koreensis*	1	100%
Endocarp	*Proteobacteria*	*Pseudomonas sp.*	1	94.61%

Layer	Phylum	Molecular Identification 16S	Number of Isolates	Similarity % NCBI
Endocarp	*Firmicutes*	*Staphylococcus sciuri*	1	99.68%
Endocarp	*Firmicutes*	*Terribacillus goriensis*	2	100.00%
Endocarp	*Firmicutes*	*Terribacillus saccharophilus*	4	93.36%
		Total Strains	***49***	

Table 4.6.2. List of the species of microbes isolated from the cacao variety CATONGO (Susceptible). Putative identification was performed amplifying the gene 16S rRNA. The potential role is provided based of scientific reports.

Layer	Phylum	Molecular Identification 16S	Number of Isolates	Similarity % NCBI
Seeds	*Proteobacteria*	*Alphaproteobacterium*	1	99.44%
Seeds	*Proteobacteria*	*Brevundimonas lenta*	1	100%
Seeds	*Proteobacteria*	*Brevundimonas sp*	1	98.93%
Seeds	*Proteobacteria*	*Devosia sp*	1	99.64%
Seeds	*Proteobacteria*	*Enterobacter sp*	1	98.76%
Seeds	*Proteobacteria*	*Erwinia sp*	1	99.30%
Seeds	*Proteobacteria*	*Gammaproteobacterium*	1	98.99%
Seeds	*Proteobacteria*	*Klebsiella oxytoca*	1	92.16%
Seeds	*Proteobacteria*	*Kluyvera ascorbata*	2	99.89%
Seeds	*Actinobacteria*	*Microbacterium sp*	1	99.40%
Seeds	*Firmicutes*	*Paenibacillus sp.*	1	96.31%
Seeds	*Proteobacteria*	*Pantoea anthophila*	1	99.13%
Seeds	*Proteobacteria*	*Pantoea rodasii*	1	99.07%
Seeds	*Proteobacteria*	*Sphingomonas koreensis*	1	98.62%
Seeds	*Firmicutes*	*Staphylococcus sciuri*	1	98.57%
Seeds	---	*Uncultured bacteria*	1	99.50%
Pulp	*Proteobacteria*	*Agrobacterium sp*	1	98.40%
Pulp	*Proteobacteria*	*Enterobacteriaceae bacterium*	2	98.68%
Pulp	*Proteobacteria*	*Erwinia billingiae*	1	96.91%%
Pulp	*Proteobacteria*	*Erwinia persicina*	1	95.68%
Pulp	*Proteobacteria*	*Klebsiella oxytoca*	1	100%
Pulp	*Proteobacteria*	*Kluyvera sp.*		99.83%
Pulp	*Firmicutes*	*Paenibacillus lautus*	1	99.22%
Pulp	*Proteobacteria*	*Pantoea anthophila*	1	98.74%
Pulp	*Proteobacteria*	*Pantoea dispersa*	1	90.97%
Pulp	*Proteobacteria*	*Pantoea rodasii*	1	98.83%
Pulp	*Proteobacteria*	*Rosenbergiella epipactidis*	1	99.83%
Pulp	*Firmicutes*	*Terribacillus gorienses*	1	99.19%
Pulp	*Firmicutes*	*Terribacillus saccharophilus*	2	92.54%
Endocarp	*Firmicutes*	*Bacillus idriensis*	1	93%
Endocarp	*Protoebacteria*	*Brevundimonas sp*	2	97.28%
Endocarp	*Protoebacteria*	*Enterobacter sp*	1	98.67%
Endocarp	*Protoebacteria*	*Kluyvera sp.*	1	99.83%
Endocarp	*Protoebacteria*	*Pantoea anthophila*	1	99.10%

Layer	Phylum	Molecular Identification 16S	Number of Isolates	Similarity % NCBI
Endocarp	*Protoebacteria*	*Pseudocitrobacter anthropi*	1	98.87%
Endocarp	*Protoebacteria*	*Pseudomonas sp*	1	98.89%
Endocarp	*Firmicutes*	*Terribacillus goriensis*	1	99.79%
		Total	***44***	

Table 4.6.3. Role of the endophytic bacteria isolated from cacao layers reported in other plant species.

Genus/Species	Role	Reference
Acinetobacter johnsonii	Plant Growth Promotion	Shi et al., 2011
Acinetobacter oleivorans	Abiotic Stress	Gkorezis et al., 2015
Acinetobacter sp	Plant Growth Promotion	Long et al., 2008
Agrobacterium sp	Plant Growth Promotion	Abbamondi et al., 2016
Alphaproteobacterium	Plant Growth Promotion	Franche et al., 2009
Bacillus cereus	Plant Growth Promotion	Hu et al., 2017
Bacillus idriensis	Plant Growth Promotion	Afzal et al., 2016
Brachybacterium conglomeratum	Plant Growth Promotion Salinity stress	Barnawal et al., 2016
Brevundimonas lenta	No identified	Yoon et al., 2007
Brevundimonas sp	Plant Growth Promotion	Manter et al., 2010
Devosia sp	Plant Growth Promotion	Rashid et al., 2012
Enterobacter cloacae	Plant Growth Promotion	Macedo-Raygoza et al., 2019
Enterobacter soli	No identified	Manter et al., 2011
Enterobacter sp	Plant Growth Promotion Plant Colonization Traits Biocontrol	Ludeña et al., 2019 Rivarez et al., 2019
Enterobacteriaceae bacterium	Plant Growth Promotion	Safiyh et al., 2010
Erwinia billingiae	Antimicrobial Traits	Jakovljevic et al., 2008
Erwinia persicina	Abiotic Stress Antimicrobial Traits	Goryluk el at., 2016
Erwinia sp	Antimicrobial Traits	Procópio et al., 2009
Gammaproteobacteria	Phytoremediation	Lumactud et al., 2016
Klebsiella oxytoca	Plant Growth Promotion	Jha et al., 2007
Klebsiella sp	Plant Growth Promotion	Govindarajan et al., 2007
Klebsiella variicola	Plant Growth Promotion	Wei et al., 2014
Kluyvera ascorbata	Plant Growth Promotion	Laurentis et al., 2014
Kluyvera sp.	Plant Growth Promotion Phytoremediation	Magnani et al., 2010
Kosakonia cowanii	Plant Growth Promotion	Panigrahi & Rath, 2019
Kosakonia sp	Plant Growth Promotion Biocontrol	Liu et al., 2017 Rivarez et al., 2019

Genus/Species	Role	Reference
Microbacterium sp	Abiotic Stress	Rajkumar et al., 2009
Paenibacillus lautus	Plant Growth Promotion	Miguel et al., 2013 Liu et al., 2016
Paenibacillus sp.	Plant Growth Promotion Biocontrol	Lal et al., 2016 Rybakova et al., 2016
Paenibacillus taichungensis	Plant Growth Promotion	Pandya et al., 2015
Pantoea agglomerans	Plant Growth Promotion	Feng et al., 2006
Pantoea anthophila	Plant Growth Promotion	Diaz-Herrera et al., 2016 Paul et al., 2013
Pantoea coffeiphila	No identified	de Siqueira et al., 2018
Pantoea dispersa	Biocontrol	Jiang et al., 2019
Pantoea rodasii	Plant Growth Promotion Plant Colonization Traits	Trifi et al., 2020
Pseudocitrobacter anthropi	Plant Growth Promotion	Parthasarathy et al., 2018
Pseudomonas cedrina	Plant Growth Promotion	Goryluk-Salmonowicz et al., 2018
Pseudomonas koreensis	Plant Growth Promotion	Liaqat & Eltem, 2016
Pseudomonas sp	Plant Growth Promotion Abiotic Stress	Win et al., 2018
Rosenbergiella epipactidis	No identified	Lenaerts et al., 2014
Rosenbergiella sp	Biocontrol Plant Growth Promotion	Lenaerts et al., 2017 Caneschi et al., 2018
Sphingomonas koreensis	Abiotic Stress	Heshman et al., 2014
Staphylococcus sciuri	Plant Growth Promotion Antimicrobial Traits	Singh et al. 2017 Alijani et al., 2019
Tatumella ptyseos	Plant Colonization Traits	Torres et al., 2008
Terribacillus goriensis	Plant Growth Promotion Plant Colonization Traits	Dent & Del Castillo Madrigal, 2016 (Patent).
Terribacillus saccharophilus	Plant Colonization Traits Stress Tolerance	An et al., 2007 Christakis et al., 2020
Terribacillus sp	Plant Colonization Traits Stress Tolerance	Sharma et al., 2014

4.7. Figures

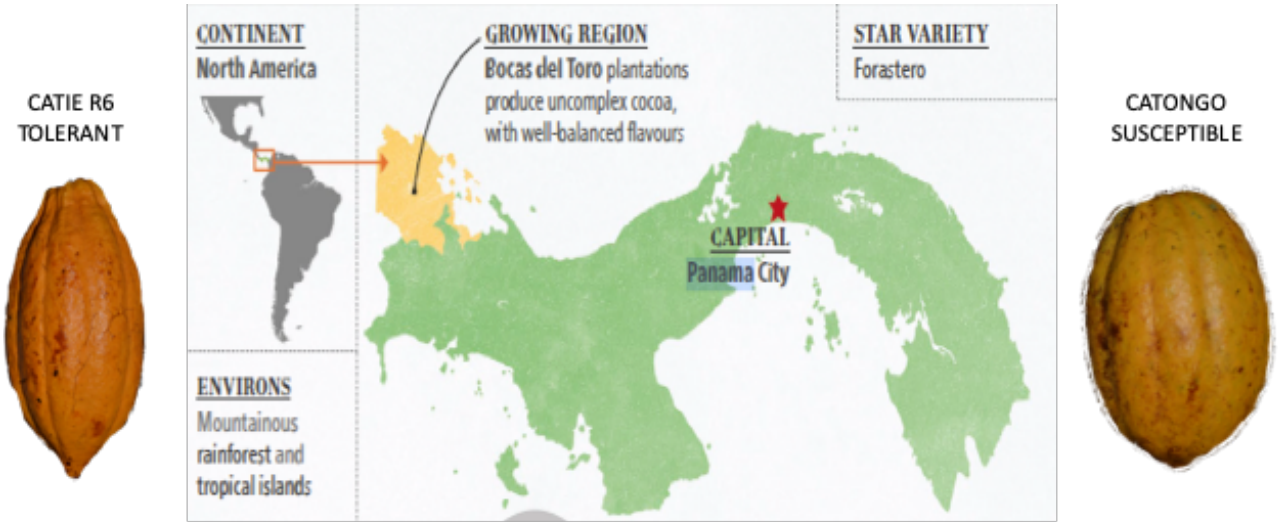

Figure 4.7.1. Map of the Republic of Panama, the yellow area represents the province of Bocas del Toro, where is located the Magnita Farm (collection site), (Ramsey, 2016).

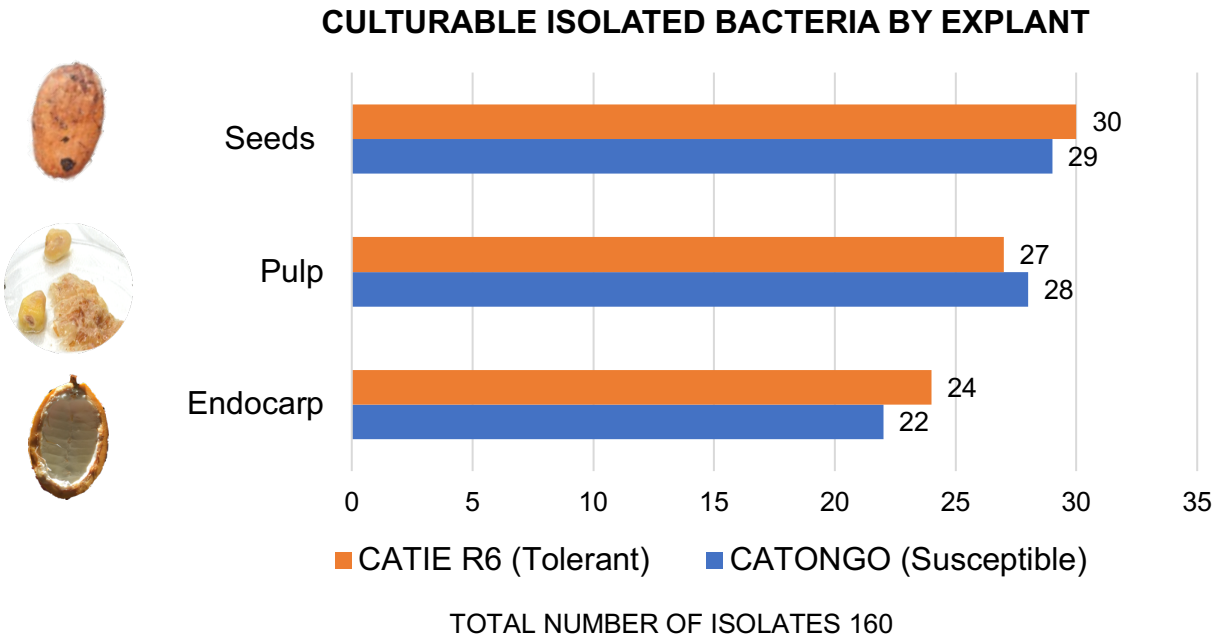

Figure 4.7.2. Number of culturable bacteria isolated from three different tissue layers of the fruits from two commercial varieties of cacao, CATONGO and CATIE-R6.

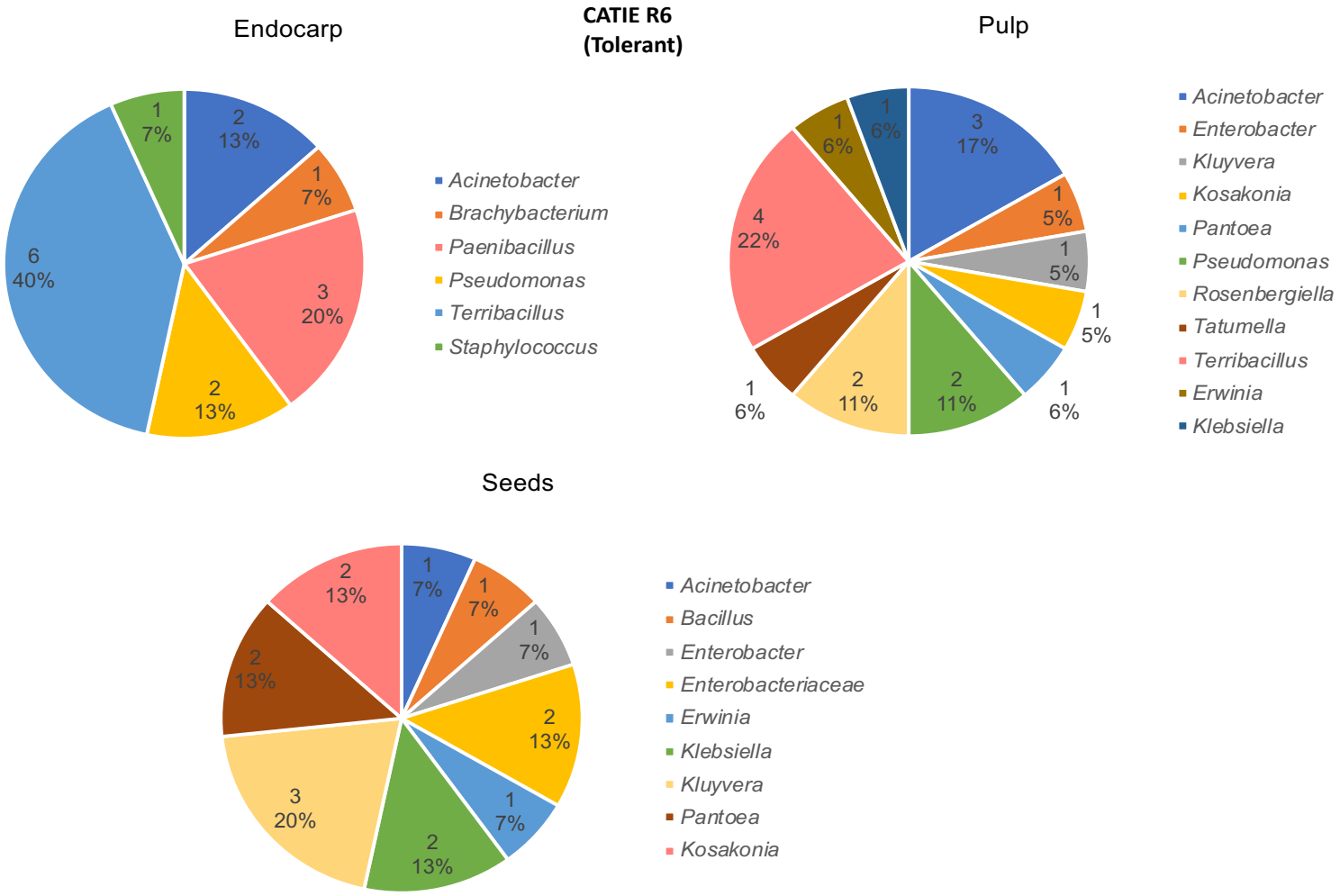

Figure 4.7.3. Microbial community isolated from seeds, pulp and endocarp from the commercial cacao variety CATIE-R6. Total of bacteria identified 48.

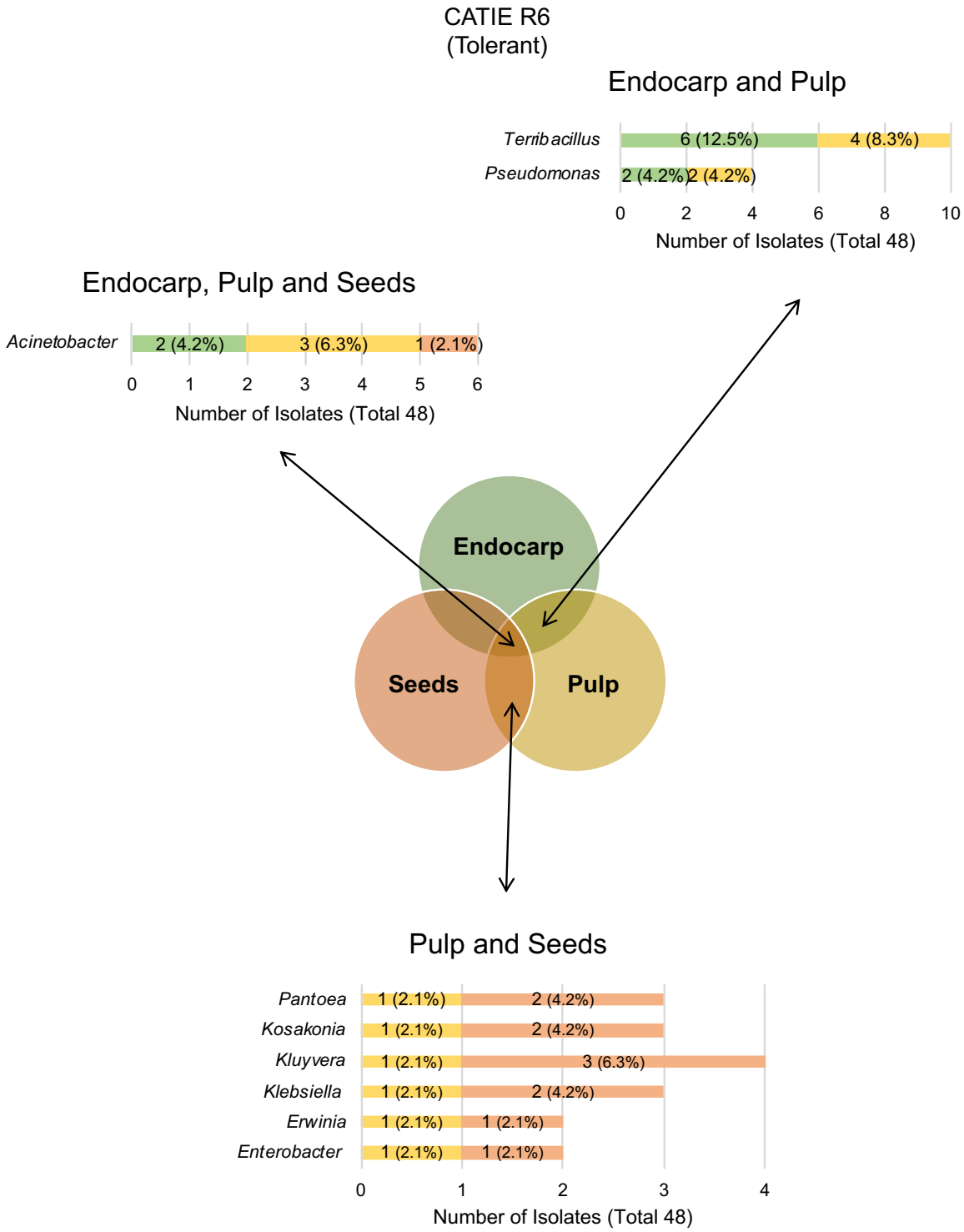

Figure 4.7.4. Endophytic bacteria isolated from the cacao variety CATIE-R6 (Tolerant). The Venn diagram shows the genera of bacteria that are present in more than one layer.

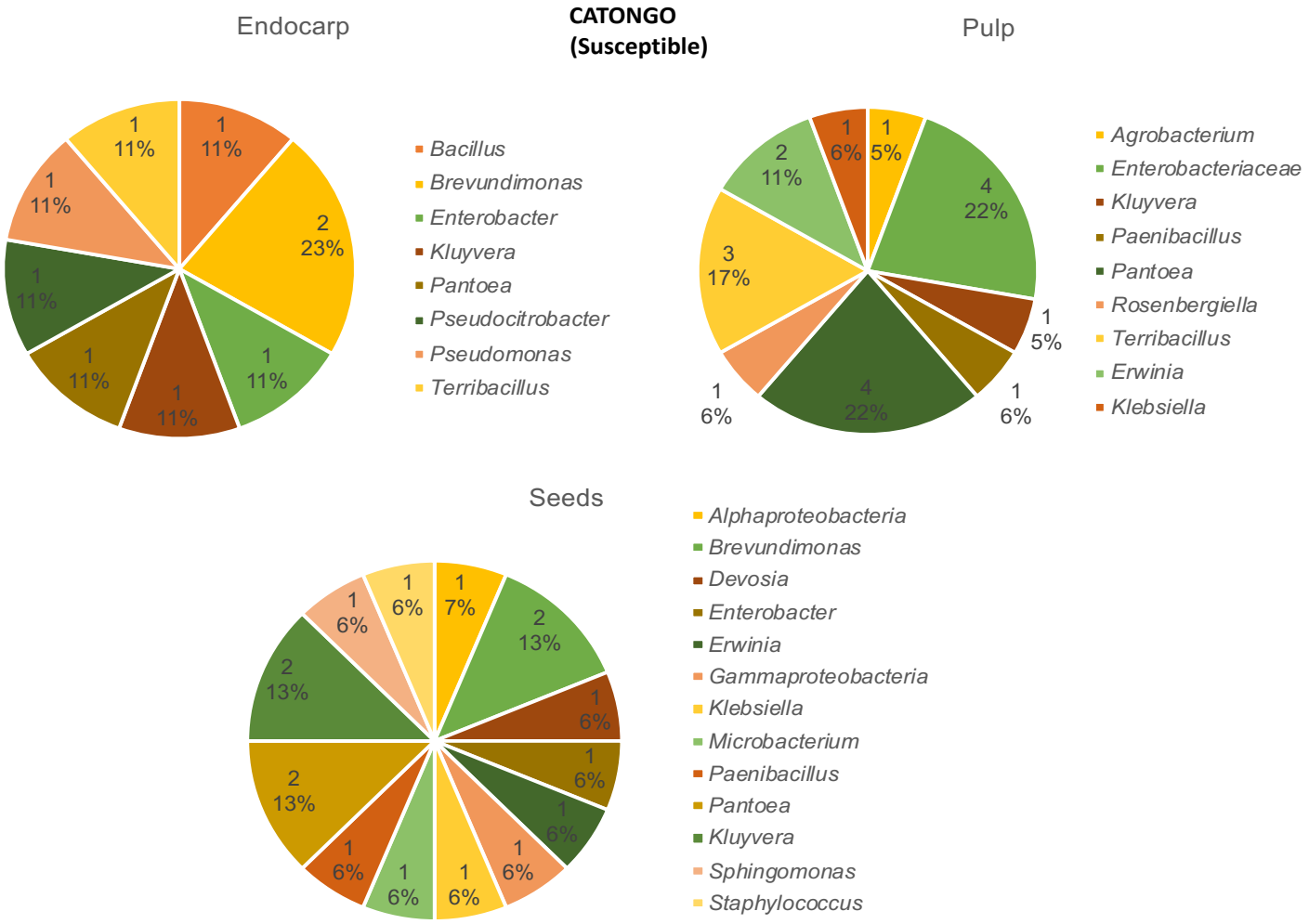

Figure 4.7.5. Microbial community isolated from seeds, pulp and endocarp from the commercial cacao variety CATONGO. Total of identified bacteria 44.

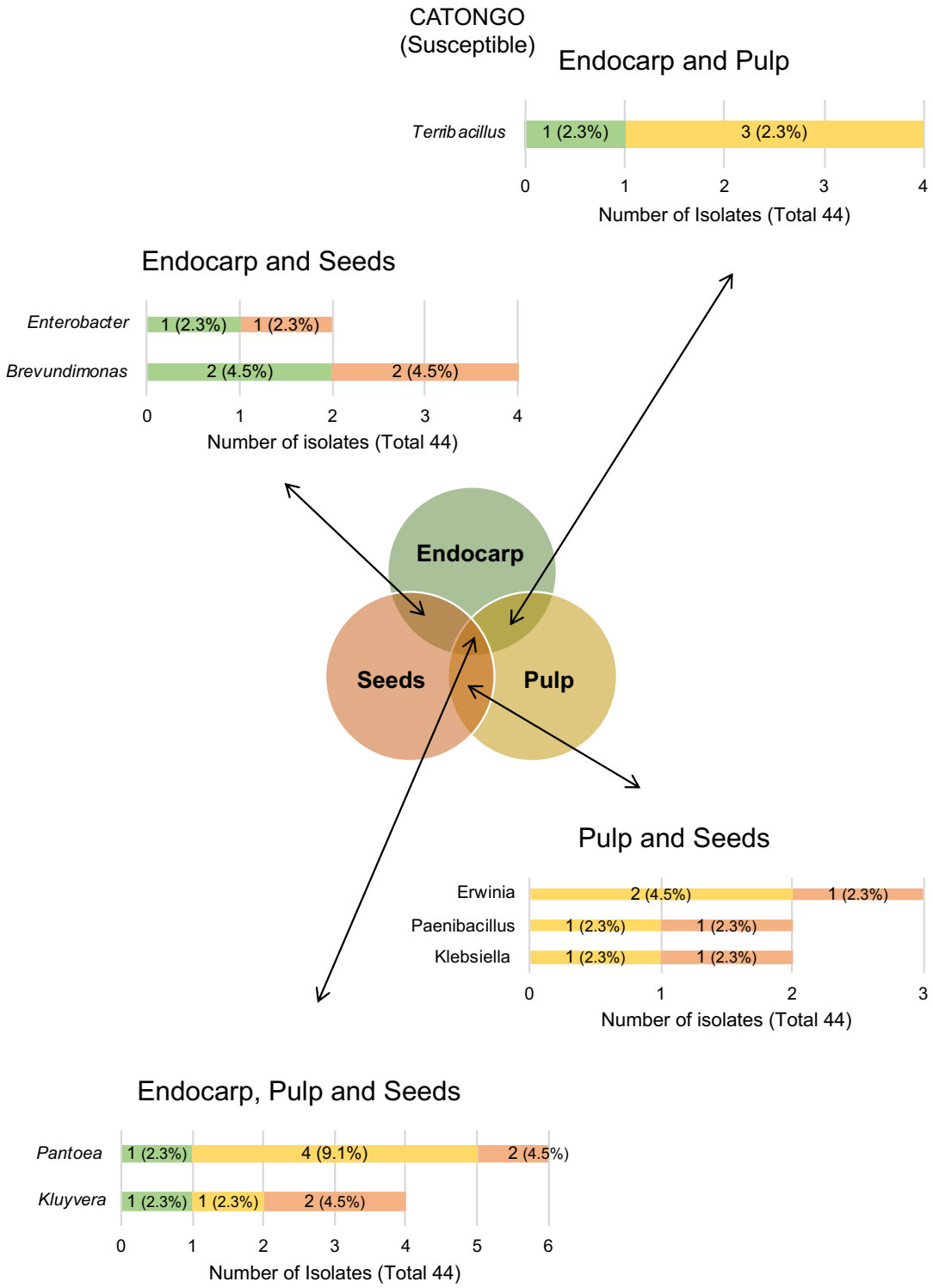

Figure 4.7.6. Endophytic bacteria isolated from the cacao variety CATONGO (susceptible). The Venn diagram shows the genera of bacteria that are present in more than one layer.

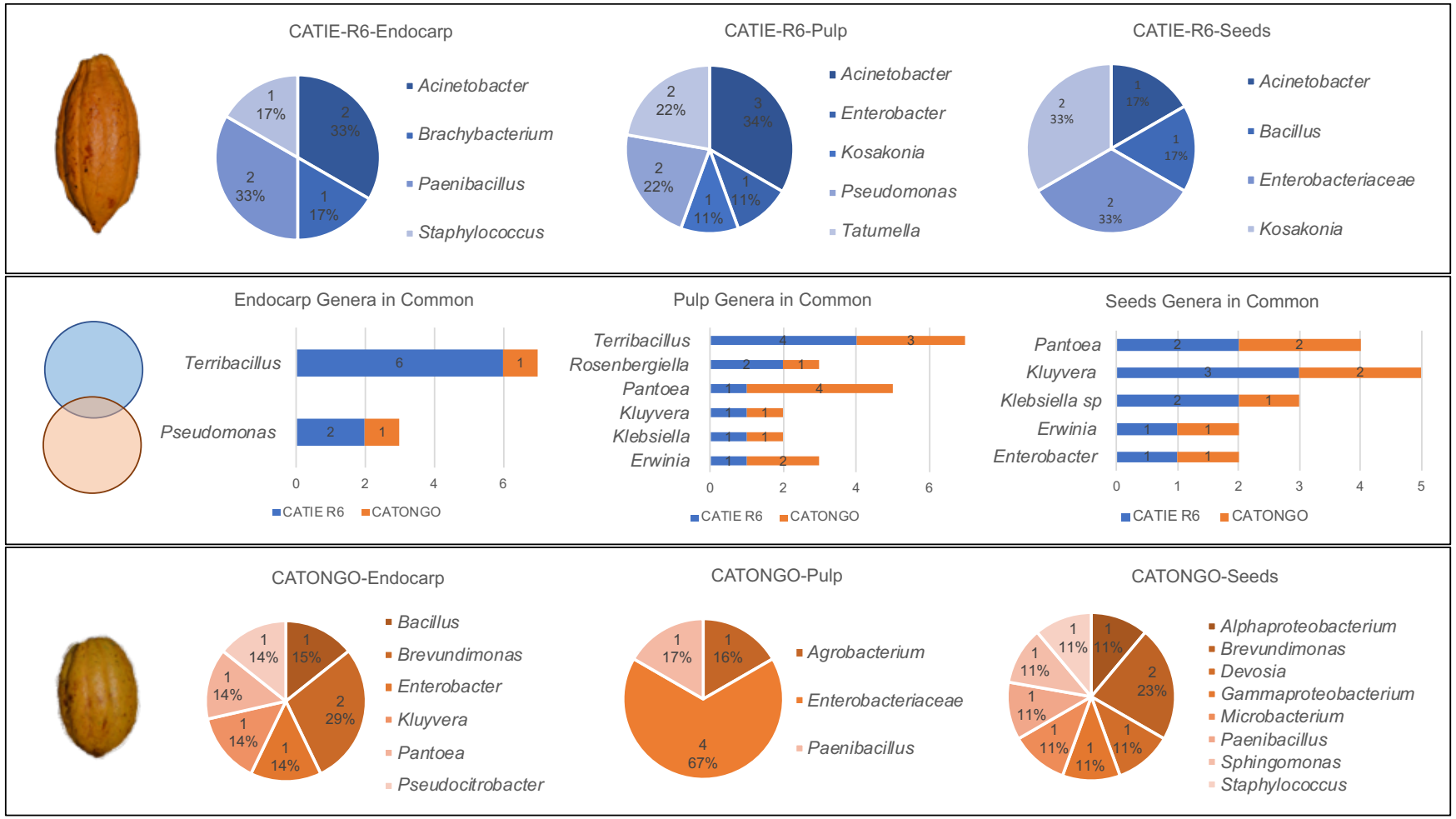

Figure 4.7.7. First row shows the unique genera of bacteria isolated from the variety CATIE-R6 (Tolerant). Second row illustrate the genera that common in both varieties. Total of morphotypes identified: 93. Third raw unique genera of bacteria found in the variety CATONGO (Susceptible).

Chapter 5

5. DISCUSSION

The global objective of this study consisted the examination of the bacterial microbiome of commercially grown varieties of cacao varying in their tolerance to frosty pod rot disease. Strategically, we were interested in examining the maximum diversity of culturable microbes isolated from the cacao fruit tissues grown on different microbiological culture media with different sources of carbon.

This approach may not support the isolation of fastidious microbial endophytes that are slow-growing or unable to grow. Next-generation sequencing can provide a full assessment of the abundance of unculturable endophytes isolated from cacao fruit (Wolińska, 2019).

Earlier studies focused on endophytic fungi in *cacao*, reported that they are vastly diverse, and show some degree of host affinity (Mejia et al., 2008; Arnold et al., 2000; Van Bael et al., 2005). For instance, cacao, leaves, and fruits are devoid of endophytes at emergence, but they acquire diverse endophytes from spore and rain from the environment. Cacao leaves become heavily colonized in a short time by a group of endophytic species characterized by dominant members of the assemblage and a large number of rare endophyte species (Arnold et al., 2003, Herre et al., 2005). A total of 344 morphotaxa were identified, being the most frequent infections generated by *Colletotrichum sp., Fusarium / Nectria spp.* and *Xylaria sp*. In another study, a total of 150 fungi were isolated from the stems of healthy and diseased trees from cacao-producing farms in Brazil. These belong mainly to the Ascomycetes group with the most frequent families are *Botryosphaeriaceae, Valsaceae, and Nectriaceae* families. *Fusarium spp*. was the dominant genus and showed the highest diversity Rubini et al., (2005).

Recently, several reports investigated the microbial community of cacao bean spontaneous fermentation in the Amazon (Serra et al., 2019), and Ghana (Camu

et al., 2007; Daniel et al., 2009; Nielsen et al., 2007); Colombia (Delgado-Ospina et al., 2020), Ivory Coast (Soumahoro et al., 2020) and West Africa (Jespersen et al., 2005).

The microorganisms that are part of the fermentation process are yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), spore forms, and Molds (Camu et al., 2007; Schwan & Wheals, 2004). Ouattara et al., (2017) assessed the LAB community from samples taken directly from the heap fermentations of six different regions on the Ivory Coast. They reported that the species *Lactobacillus plantarum* and *Leuconostoc mesenteroides* were recovered from all the locations. However, the microbial composition is different among the locations assessed. Several factors might play a role in the microbial population, like the concentration of polysaccharides in the pulp, which rely on cacao genotypes, the quality of the soil, and environmental conditions (Quattara et al., 2017; Pereira et al., 2012).

Similarly, in Colombian cacao farms, a metagenomic study was performed where they wanted to know the impact of farm protocols, climate and bean mass exposure in the dynamics and composition of the microbial communities. To do that, every 12 hours they collected pulp samples placed inside of a wooden fermentation box. The samples were frozen to do metagenomics and establish the dominant microbial communities during the dynamic of the microbial fermentation. Some of the results point out that the genus *Enterobacteriaceae* was detected during the early stages of the process, because this group of bacteria has biochemical properties like degradation of pectin and assimilation of citrate (Pacheco-Montealegre et al., 2020). Likewise, during the first hours were found the genera *Pantoea*, *Enterobacter*, and *Tatumella*, these genera are common inhabitants of the plant tissues, that could support the fact of their early emergence in the initial steps of the fermentation. Besides, species like *Enterobacter cloacae* has been described by Leite (et al., 2013) as a common colonizer of healthy plants (Pacheco-Montealegre et al., 2020).

Taken together and to the best of our knowledge, this study describes the first report on the abundance, diversity, and distribution of bacterial endophytes associated with various tissues of two cacao varieties originating from the Republic of Panama and varying in their tolerance against frosty pod rot, and bacterial endophytes associated with Peruvian fermented cacao seeds.

This study demonstrated that Peruvian cacao fermented seeds are associated with a limited assemblage of culturable bacteria that exist as endophytes and belong to six taxonomic groups (*Bacillus*, *Paenibacillus*, *Klebsiella, Enterobacter, Entercoccus,* and *Satphylococcus*, with *Bacillus,* constituted the most common and most diverse genus (i.e., 12 species). Strains of *Bacillus* (Park et al., 2017)*, Klebsiella* (Govindarajan et al. 2007)*, Enterobacter* (Macedo-Raygoza et al., 2019), and *Enterococcu*s (Martínez-Rodríguez et al., 2014) are known as growth-promoting bacteria. This agrees with the study of Melnik et al., (2011) who reported that the most abundant taxon encountered in cacao explants were *Bacillus* and *Paenibacillus* species. Likewise, the composition of the microbial community associated with seeds belonging to different plant species, shows that the pool of endophytic bacteria is not very variable among species as it is driven by host genotype (Frank et al., 2017).

Generally, the most common strains associated with seeds belonged to *Bacillus, Pseudomonas, Paenibacillus, Micrococcus, Staphylococcus, Pantoea, and Acinetobacter* (Truyens et al., 2015). In agreement with these studies, some of the same genera were identified in the Peruvian seeds and seeds of cacao varieties of Panama. Contrast to previous studies on cacao bean spontaneous fermentation, we did not isolate yeasts, lactic acid bacteria (LAB), or acetic acid bacteria (AAB) regardless of several attempts using enriched microbiological media that favors such groups. The reason could be due that the fermentation process is a continuous succession of three different groups of microbes. The shift of these species during the process is driven by factors like the availability of a particular substrate and temperature. Once each step is finished after a certain number of hours, a specific population of microorganisms are reduced and disappeared (Schwan & Wheals, 2004).

Seed-borne endophytes are essential because they are passed between successive plant generations via vertical transmission, thus ensuring their presence in the next generation of seedlings (Shahzad et al., 2018). Seed endophytes have been reported to be transmitted through three different pathways: (i) via xylem tissues in the maternal plant; (ii) through floral pathways, via the stigma and (iii) external colonization by the environment (Shahzad et al., 2018). Vertical transmission is reported probably to be a widespread phenomenon in prevalent endophytes. Gagné-Bourgue (et al., 2013)

detected vertical transmission of the species of endophytes *Bacillus* and *Microbacterium spp*. which were isolated from the generation F1 and F2 from seeds of the plant switchgrass. On the other hand, a study in *A. thaliana* suggests that the plant may select seed endophytes based on environmental stressors and pass them on to the next generation (Frank et al., 2017). In the case of the cacao fruit, no reports exist on how the endophytes are transmitted to the next generation.

Most of the seed-borne bacterial endophytes utilize direct or indirect mechanisms to improve plant growth and enhance plant tolerance to biotic and abiotic stresses (Afzal et al., 2019; and Shahzad et al., 2017). The ability of endophytes to produce plant hormones, HCN, and siderophores is an attractive trait that makes the microorganisms successful competitors in different environments (Loaces et al., 2011). For instance, the production of IAA promotes plant development; siderophores and HCN enhance the soil structure, and help in bioremediation of contaminated soils by sequestering heavy metals. In this study **(Chapter 3)**, the endophytes associated with the Peruvian seeds including, *Bacillus, Klebsiella, Enterobacter, and Enterococcus* exhibited more than one of the above traits. Future studies aimed at the application of the already mentioned microbes as multi-species consortia instead of single species in cacao plantations could be very effective as natural fertilizer agents and thus help mitigate the environmental impact of the use of agrochemicals (Altendorf, 2017).

Around 60% of the endophytes associated with the Peruvian seeds belonged to the genus *Bacillus*. Bacteria strains belonging to that genus make excellent candidates as biological control agents due to the following traits: they are able to form spores, which are resistant to desiccation, they produce antimicrobial compounds and lytic enzymes (Lyngwi & Joshi, 2014), (Logan & Vos, 2015). Recently, *B. subtilis strain* (ALB 629) was able not only to control in vitro the pathogen *Moniliopthora perniciosa* in cacao plants but also was to promote growth (Falcäo et al., 2014).

Tissue type plays an important role in endophytic colonization (Compant et al., 2010). Although there are no descriptive studies of the abundance and diversity of culturable bacteria from the internal layers of the cacao fruits (i.e., pulp and endocarp), Tchinda (et al., 2016) described the actinobacterial diversity present in the mesocarp and the seeds of the cacao fruits harvested in Cameroon. The proportion of the actinobacteria

was higher in the seeds in comparison to the mesocarp. Also, from the endocarp were isolated nine morphotypes that were absent in the endocarp, confirming that endophytic communities vary within plant structures.

In this study, specific genera of bacteria associated with a specific tissue and variety were found. Most of the microbes isolated could play a potential role as growth promoters as it was described in **Table 4.6.3**. However, in the tolerant variety CATIE-R6, the species *Staphylococcus sciuri* was isolated from the endocarp. The strain *Staphylococcus sciuri* MarR44 is a potential biofumigant that inhibited the growth of the *Colletotrichum nymphaeae* the causal agent of strawberry anthracnose under in vitro and field conditions (Alijani et al., 2019). Another example is the unique presence of *Kokasonia* in the CATIE-R6 variety in the pulp and seeds. Interestingly, *Kosakonia* strain EBW was isolated from a tolerant papaya genotype to *Erwinia mallotivora* (Rivarez et al., 2019) Inoculated papaya seeds with *Kosakonia* strain EBW stimulated seed germination of different papaya genotypes and also reduced disease severity caused by *Erwinia* (Rivarez et al., 2019). Interestingly, the two genera *Brachybacterium* and *Tatumella* were specific to the tolerant variety CATIE-R6. *Brachybacterium* strains are associated with the enhancement of crop tolerance against salinity stress (Barnawal et al., 2016). While *Tatumella* strains are usually present in the initial steps of the fermentation process of cacao seeds (Pacheco et al., 2020).

Remarkably, in our study, the genus *Acinetobacter* was isolated from all layers of the tolerant variety. It was reported that *Acinetobacter* strain LCHoo1 isolated from the plant *Cinnamomum camphora* releases three antifungal metabolite compounds which inhibited the growth of several phytopathogenic fungi such as *Cryphonectria parasitica*, *Glomerella glycines*, *Phytophthora capsici*, *Fusarium graminearum*, *Botrytis cinerea*, and *Rhizoctonia solani* (Liu et al., 2007).

It would be interesting to detect if the genus *Acinetobacter* is still the dominant microorganism when the tolerant variety is subjected to the infection by *M. roreri*, as it was reported in citrus fruits, the study carried out by Shahzad (et al., 2020) in citrus fruits. They isolated endophytes of symptomatic and asymptomatic citrus varieties, determining that the *Bacillus sp* species was dominant and only isolate from tolerant plant material, they assumed that this strain may be responsible for tolerance to pathogen infection.

However, the *Curtobacterium* species was isolated from the leaves of tolerant varieties but in a lower proportion. Based on bibliographic records, this species has antagonistic traits against pathogens, but its efficacy was not tested in the laboratory. They hypothesize that the synergy between *Bacillus* and *Curtobacterium* may enhance the response of the plant towards the pathogen or in the production of antimicrobials.

On the other hand, genera like *Paenobacillus*, *Enterobacte*r, and *Enterobacteriaceae* were isolated from both varieties but they were being associated with different tissues. Among the genera that were common in the same tissue of both varieties is *Pseudomonas* which was associated with the endocarp and, *Kluyvera, Pantoea,* and *Klebsiella* which was found in the pulp. Accumulated evidence supported that species belonging to the above-mentioned genera commonly isolated from several plant species, making these taxonomic groups the dominant core of endophyte communities of cultivated plants worldwide. In most of the cases, these bacterial genera supported the development of the growth of the host plants **(Table 4.6.3)**, (Hallman, 2001), (Muthu Kumar et al., 2017).

We were intrigued to report that the susceptible variety CATONGO had a greater diversity of endophytes associated with the seeds. Cacao seeds are rich in bioactive secondary compounds such as phenols (e.g., gallic acid, catechin, and epicatechin) (Scapagnini et al., 2014). Additionally, phenolic compounds play a major role in the induction of resistance in plants and phenolic compounds released from seeds and roots can act against soil borne-pathogens (Mandal et al., 2010; Gouda et al., 2016). CATONGO seeds have low proportions of theobromine and phenolic compounds. Accordingly, it is described to have a low intensity of bitterness, acidity, and astringency flavor of chocolate, which is more acceptable for the public (Das Virgens et al., 2020). The low concentrations of the phenolic compounds could explain why CATONGO seeds are associated with a greater diversity of bacteria.

We were not able to conduct functional biochemical traits on the 93 morphotypes isolated from different tissues of the two cacao varieties (CATIE-R6 and CATONGO). The reason being that live cultures of the bacterial isolates were stored in Panama at the Smithsonian Tropical Research Institute (STRI), and the import permit was delayed due to the COVID-19 pandemic situation.

Chapter 6

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The scope of this thesis was to mine the bacterial endophytic community associated to the cacao fruits, with the main focus on selective microorganisms associated with plant growth promotion and antagonistic activity effect. To accomplish this objective, cultivable bacteria from two-layers of the fruits and seeds from two cacao varieties harvested in Panama. with different levels of susceptibility to the pathogen *M. roreri* were evaluated. Similarly, the isolation of bacteria from Peruvian organic cacao seeds obtained commercially in Montreal was carried out. After performing the isolation, identification, and biochemical tests, the following conclusions for the development can be drawn:

Firstly, the two varieties of cacao that were evaluated show a core of common microorganisms between them and with other plant species of commercial interest. Most of these genera of bacteria have been classically linked to growth-promoting processes. However, a low proportion of unique bacteria species were also found in each variety, which is implicit in the resistance processes against pathogens, specifically in the case of the tolerant variety (CATIE-R6).

Secondly, the group of bacteria isolated from the Peruvian organic cacao seeds, correspond in greater proportion to the genus *Bacillus*, all the isolates of this genus exhibited biochemical characteristics that promote the plant growth. In a smaller proportion, *Klebsiella* strains that were isolated can synthesize the highest concentration of IAA among all the strains evaluated (324.38 µg / ml).

Based on the results obtained, the following strains: *Bacillus aerius, Enterobacter tabaci, Enterobacter spp, E. tabaci, Klebsiella spp.* and *K. varicola* were outstanding in their performance in different assessed tests (i.e., IAA, siderophore, HCN). It would be very useful to evaluate the efficacy of these isolates as plant growth promoters. These strains could be evaluated individually or in consortia, initially observing their effect on seed germination (Ali et al., 2009).

Thirdly, it has been recorded that *Klebsiella* (Mendoza-Hernandez et al., 2016) and *Enterobacter* (Ojuederie & Babalola, 2017) strains can help in the bioremediation process of the soils. This problem is of current interest in cacao because the exportation of the seeds has been compromised due to the high concentration (>0.80mg/kg) of cadmium in the raw material used to manufacture the chocolate (Arévalo-Gardini et al., 2017), (Official Journal of the European Union, 2014). Most of the commercial cacao varieties bioaccumulate the cadmium taken from the soil in the seeds. Efforts to mitigate this problem have been directed towards obtaining improved varieties that bioaccumulate cadmium in vegetative parts of the plant, or the use of mycorrhizal fungi associated with cacao crops with bioaccumulative properties. However, these solutions have not been so fruitful so far (Meter et al., 2009). In this work, *Klebsiella* and *Enterobacter* strains were isolated with the traits that indicate that they could potentially be useful in the bioremediation process, which is the production of metabolites related to growth promotion, like IAA and siderophores (Mendoza-Hernandez et al., 2016).

We have reported the bacterial microbiome of two layers from the fruits and seeds of one tolerant (CATIE-R6) and one susceptible variety of cacao (CATONGO). The results show that some endophytes are unique and other ones are common between the two varieties. Intensive screening of several varieties of cacao with different grades of tolerance to frosty pod rot would be valuable, to discover if there is any pool of microorganisms associated with the resistance to frosty pod rot (Purahong et al., 2018).

The future experiment should aim at evaluating the diversity of antagonistic microorganisms that could potentially reduce the incidence of frosty bud rot (Liu et al., 2007).

Chapter 7

7. BIBLIOGRAPHY

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Chapter 8

APPENDICES

Appendix I. List of culture media used to isolate endophytic bacteria associated with cacao seeds*

Media	Composition (Per Liter)
DeMan-Rogosa and Sharpe Agar (MRS Agar)	Diammonium citrate, 2 g, Dipotassium hydrogen phosphate 2 g, Glucose, 20 g, Magnesium sulfate heptahydrate, 0.2 g, Manganous sulfate tetrahydrate 0.05 g, Meat extract 10 g, Meat peptone (peptic) 10 g, Sodium acetate trihydrate 5 g, Yeast extract 5 g, Agar 12 g.
Tryptic Soy Broth (TSB)	Pancreatic Digest of Casein 17 g, Sodium Chloride 5 g, Papaic digest of soybean meal 3 g, Dextrose 2.5 g Dipotassium phosphate 2.5 g.
Luria-Bertani Agar (LBA)	NaCl 10 g, Peptone 10 g, Yeast 5 g, Agar 12 g.
Malt extract agar (MEA)	Malt Extract 30 g, Peptone 5 g, Agar 15 g.
Nutrient Agar (NA)	Nutrient Broth 4 g, Glucose 1 yeast Extract 1g, Agar 15 g.
Potato Dextrose Agar (PDA)	Dextrose 20 g, Potato Extract 4 g, Agar 15 g

*All chemicals purchased from Sigma Aldrich.

***Appendix* II.** Biochemical tests performed on isolated endophytic bacteria.

Plant Colonization Traits	Growth Promotion Traits	Antimicrobial Traits
Substrate solubilization	Phytohormones and others	Releasing metabolites
Amylases	IAA	Antibiotics
Cellulases	Organic acids	Chitinases
Proteases	Calcium Solubilization	Siderophores
Alginate Lyases	ACC deaminase	HCN
		Confrontation Assay

Appendix III. Composition of culture media used to determine the ability of endophytic bacteria to produce the listed lytic enzymes.

Test	Composition
Amylase (Lugol-based)	Peptone 0.5% Yeast 0.25% Starch 0.25% Agar 1.5% (Saleh et al. 2019).
Alginate-Lyase (Lugol-based)	Peptone 0.05%, Yeast 0.03%, Na-Alginate 0.2%, $(NH_4)_2SO_4$ 0.2%, KH_2PO_4 0.1%, $MgSO_4$ $7H_2O$ 0.05%, Agar 1.5.
Cellulase (Lugol-based)	Peptone 0.5%, Yeast 0,25%, Glucose 0.1%, Carboxymethyl cellulose 1.0%, Agar 1.5%
Protease	Skim milk 2.8%, Casein 0.5%, Yeast Extract 0.1%, Agar 1.5% (Saleh et al. 2019).
Lugol Solution (Revealed Solution)	1 g of Iodine crystal, 2 g of Potassium iodine, 300 mL of sterile distilled water

***Appendix* IV.** Stock solutions for the preparation of the mediums DF 1 and 2. ACC deaminase test.

DF Minimal Medium					
Part 1 Dissolve in **100ml** of water the following components, this solution can be it storage in the refrigerator up to several months.		**Part 2** Dissolve in **10ml** of water the following component, this solution can be it storage in the refrigerator up to several months		**Part 3*** Dissolve in **1 L** of water the following components. Moreover add 0.1ml of the solution Part 1 and Part 2.	
H_3BO_3	10mg	**$FeSO_4 * 7H_2O$**	100mg	**K_2HPO_4**	4g
$MgSO_4 * H_2O$	11.19mg			**Na_2HPO_4**	6g
$ZnSO_4 * 7H_2O$	124.6mg			**$MgSO_4 * 7H_2O$**	0.2g
$CuSO_4 5H_2O$	78.22mg			**Glucose**	2g
MoO_3	10mg			**Gluconic Acid**	2g
				Citric Acid	2g
				$(NH_4)_2SO_4$	2g

*The solution 3 must be autoclaved for no more than 20 minutes. If you decided to prepare it by dissolving one ingredient at a time, namely but no adding another ingredient until the first one is completely dissolved, the final preparation should not contain a precipitate.

- **DF Salts minimal medium 1**

Mix the elements of the part 3 and 0.1mL of the solution Part 1 and Part2.

- **DF Salts minimal medium 2**

Mix elements part 3 and 0.1mL of the solution Part 1 and Part 2. DO NOT ADD $(NH_4)_2SO_4$. Add 45 µL of 0.5 M of 1-Aminocyclopropane 1-Carboxylic Acid **(**ACC) solution.

Appendix V. Stocks and working solutions of tested antibiotics.

Antibiotic	***Stock Concentration***	***Working Concentration***	***Dissolve***
Ampicillin	50 mg/mL	50 µg/mL	Water MiliQ
Chloramphenicol	25 mg/mL	34 µg/mL	EtOH 100%
Kanamycin	10 mg/mL	50 µg/mL	Water MiliQ
Rifampicin	50 mg/mL	100 µg/mL	Methanol 100%
Streptomycin	50 mg/mL	50 µg/mL	Water MiliQ