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Identification and characterization of Vietnamese coffee bacterial endophytes displaying *in vitro* antifungal and nematicidal activities

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ABSTRACT

The endophytic bacteria were isolated from coffee roots and seeds in Vietnam and identified with 16S rDNA sequencing as belonging to the Actinobacteria, Firmicutes and Proteobacteria phyla with the *Nocardia*, *Bacillus* and *Burkholderia* as dominant genera, respectively. Out of the thirty genera recovered from *Coffea canephora* and *Coffea liberica*, twelve were reported for the first time in endophytic association with coffee including members of the genera *Brachybacterium, Caballeronia, Kitasatospora, Lechevalieria, Leifsonia, Luteibacter, Lysinibacillus, Mycolicibacterium, Nakamurella, Paracoccus, Sinomonas* and *Sphingobium*. A total of eighty bacterial endophytes were characterized *in vitro* for several plant growth promoting and biocontrol traits including: the phosphate solubilization, the indolic compounds, siderophores, HCN, esterase, lipase, gelatinase and chitinase production. A subset of fifty selected bacteria were tested for their potential as biocontrol agents with *in vitro* confrontations with the fungal pathogen *Fusarium oxysporum* as well as the coffee parasitic nematodes *Radopholus duriophilus* and *Pratylenchus coffeae*. The three most efficient isolates on *F. oxysporum* belonging to the *Bacillus, Burkholderia*, and *Streptomyces* genera displayed a growth inhibition rate higher than 40%. Finally, five isolates from the *Bacillus* genus were able to lead to 100% of mortality in 24 h on both *R. duriophilus* and *P. coffeae*.

1. Introduction

Coffee is one of the most consumed beverages and the most traded tropical agricultural commodity worldwide (FAO, 2018; Mussatto et al., 2011). The coffee tree is a perennial plant belonging to the Rubiaceae family. The *Coffea* genus consists of more than one hundred species, but only *C. arabica*, *C. canephora,* and *C. liberica* are used for beverage production and often referred as Arabica, Robusta and Liberica coffee, respectively (Davis et al., 2006). Millions of people, especially in developing countries, rely on coffee for their livelihood (ICO, 2019a). Vietnam is the world's second-largest coffee producer and exporter. While both *C. arabica* and *C. canephora* species are cultivated, Robusta represents more than 95% of the Vietnamese coffee production. The Central Highlands region encompasses the main producing provinces, namely the Dak Lak (190,000 ha), Lam Dong (162,000 ha), Dak Nong (135,000 ha), Gia Lai (82,000 ha) and Kon Tum (13,500 ha) with an overall cultivated area estimated at 600,000 ha. (ICO, 2019b; USDA, 2018).

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In order to maintain its position as one of the leading coffee producers and exporters, Vietnam is facing two major challenges: (i) the adoption of more sustainable farming practices in order to reduce the use of chemical inputs, and (ii) the replacement of the less productive aging coffee trees (ICO, 2019b). Around 250,000 ha of plantation are covered by trees older than 20 years that need to be replanted on the period 2017-2031. This rejuvenation is an expensive process because of the soil treatments needed to make it successful and the problems caused by nematodes and fungal pathogens (Truong, 2018). Indeed, nematodes were determined as the primary cause of Robusta coffee replanting issues, showing a failure rate of up to 70% in the absence of control measures (Chapman, 2014). Moreover, the involvement of fungal pathogens, such as *Fusarium oxysporum* in disease complexes with nematodes, has already been reported (Bertrand et al., 2000; Loang, 2002; Negron and Acosta, 1989).

Within numerous nematode species infecting coffee worldwide, *Pratylenchus* spp. (Root Lesion Nematodes, RLN) and *Meloidogyne* spp. (Root Knot Nematodes, RKN) are the most prevalent (Bell et al., 2018; Souza, 2008). In Vietnam, Trinh et al. (2009) described the nematodes parasites of *C. arabica* and *C. canephora* in eight producing provinces and reported *Pratylenchus coffeae*, *Radopholus arabocoffeae,* and *Meloidogyne* spp. in 24%, 9%, and 12% of the root samples, respectively. Furthermore, *Radopholus duriophilus* which was first described in durian (Nguyen et al., 2003) was later reported in *C. canephora* roots (Trinh et al., 2012, 2004). Recently, Hoang et al. (2020) described parasitic nematodes damaging *C. canephora* roots in the Dak Lak province and found *Meloidogyne* spp. as dominant species in 5 and 40 year-old plantations while *P. coffeae* was the dominant species in 18 year-old plantations.

The strategies to overcome coffee replanting failure involve the production of nematode free seedlings, nematode resistant cultivars, crop rotation or fallow as well as the use of chemical nematicides (Chapman, 2014; Souza, 2008). However, the efficient chemical compounds are already or are going to be banned due to their adverse effects on the environment and human health (Costa, 2006; Fuller et al., 2008; Husain et al., 2010). Furthermore, long term crop rotation and fallow are sometimes inefficient and challenging to implement for coffee smallholders due to the income losses associated (Chapman, 2014).

It is now widely recognized that some of the micro-organisms interacting with the plants are directly or indirectly beneficial through plant growth promotion mechanisms and antagonist relationships towards their pathogens (Compant et al., 2005; Olanrewaju et al., 2017). The most studied source of beneficial micro-organisms is the plant rhizosphere and the bacteria present in the soil surrounding the roots, the so-called plant growth-promoting rhizobacteria 'PGPR' (Kloepper and Schroth, 1978).

Endophytes represent another class of micro-organisms able to colonize the inner tissues of the plant without inducing negative symptoms on their host (Wilson, 1995). The endophytic bacteria can be considered as a subset of the rhizosphere population able to interact efficiently with their host (Ali et al., 2014; Germida et al., 1998). The internal colonization allows endophytes to be less affected by the edaphic conditions and the competition with other micro-organisms (Santoyo et al., 2016). Bacterial endophytes are now well recognized as prime candidates for enhancing plant growth through the phytohormones production, the ethylene level reduction, the nitrogen fixation and by increasing the availability of nutrients (Gaiero et al., 2013; Santoyo et al., 2016). The use of endophytic bacteria as biocontrol agents is also of growing interest as they colonize the same ecological niche as various phytopathogens, including fungi and nematodes (Anjum et al., 2019; Berg and Hallmann, 2006). Endophytes are able to stimulate plants defenses and to display antagonistic properties toward pathogens by producing various active compounds such as antibiotics, siderophores, hydrogen cyanide and several enzymes (De Silva et al., 2019; Eljounaidi et al., 2016).

Several studies already highlighted their capacities to solubilize phosphate (Teshome et al., 2017), to fix the atmospheric nitrogen (Jimenez-Salgado et al., 1997) and to promote the growth of coffee seedlings (Silva et al., 2012). The *in vitro* nematicidal and antifungal activities of coffee associated endophytic bacteria were demonstrated against parasitic nematodes and plant pathogenic fungi (Hoang et al., 2020; Mekete et al., 2009; Shiomi et al., 2006; Silva et al., 2012). Finally, their *in planta* biocontrol capacities against coffee parasitic nematodes (*Pratylenchus coffeae* and *Meloidogyne incognita)* and the fungal causative agent of coffee leaf rust (*Hemelia vastatrix*) were also reported by several authors (Asyiah et al., 2018; Mekete et al., 2009; Silva et al., 2012). However, the use of coffee bacterial endophytes is still poorly explored.

In the present study, we established a large collection of culturable endophytic bacteria isolated from coffee roots and seeds. We studied their potential as plant-growth-promoters and biocontrol agents against some of the major plant parasitic nematodes and fungal pathogens associated with coffee in Vietnam and worldwide. Firstly, bacterial endophytes were screened for several activities well known to be involved in plant growth promotion and biocontrol mechanisms in order to select the most promising isolates. As nematodes were reported as the primary cause of replanting failure in Vietnam (Chapman, 2014), sometimes in association with fungal pathogens (Loang, 2002), the selected bacterial endophytes were further tested for their direct antifungal and nematicidal effects on the plant-pathogenic fungus *Fusarium oxysporum* and two of the most prevalent migratory endoparasitic nematodes genera associated with coffee in Vietnam, namely, *Radopholus* and *Pratylenchus* (Trinh et al., 2009). Our results highlighted the high potential of bacterial endophytes as plant-growth-promoters and biocontrol agents.

2. Materials and Methods

2.1. Bacterial endophytes isolation and identification

2.1.1. Plant material sampling

Samples were collected from coffee fields presenting nematode infection symptoms in two cities located in the two main Vietnamese coffee-producing provinces, namely, Bao Loc (850 masl, 11° 34' 23'' N - 107° 50' 5'' E, Lam Dong, Vietnam) and Buon Ma Thuot (456 masl, 12° 39' 58'' N - 108° 2' 18'' E, Dak Lak, Vietnam) in June (vegetative growth period) and October (harvest period) 2018, respectively. In Bao Loc, roots were collected in two different fields from *C. canephora* trees either grafted (first field) or ungrafted (second field) on *C. liberica* rootstocks. In both fields, ten trees were randomly selected and roots were pooled in order to constitute a composite sample for each species. In Buon Ma Thuot, roots and ripened cherries (available at this time) were collected in one field from ten randomly selected *C. canephora* trees and pooled in order to constitute a composite sample for each organ. The plant materials were kept in plastic bags at 4 °C and processed for bacterial isolation within 24 h.

2.1.2. Bacterial isolation from roots and seeds

The young, healthy feeding roots and ripened cherries were washed thoroughly under tap water followed by 3 rinses with Sterile Saline Solution (3S) containing 0.85% (w/v) of sodium chloride under a laminar flow hood. The excess of moisture was removed on sterile filter paper. The roots without external damages were excised in a small fragment of 2-3 cm, and the seeds were retrieved by removing the exocarp (skin), the mesocarp (pulp), and the endocarp (parchment). At this step, the plant materials were surface-sterilized with sodium hypochlorite (2.5% available chlorine) for 2 min., followed by 5 rinses with 3S to remove the surface sterilizing agent. The surface-sterilized roots were crushed with sterile mortar-pestle and resuspended in 5 mL of 3S. Seeds were cut in 2-4 fragments on a sterile filter paper. The sterilized roots and seeds were then plated on Petri dishes containing Tryptic Soy Agar (TSA) medium (Merck KGaA, Darmstadt, Germany). To assess surface sterilization efficiency, the 3S from the last rinse was inoculated by flooding on control TSA containing Petri dishes in

triplicate. All the Petri dishes were incubated at 28 °C for up to 14 days. Isolates were purified, stored in glycerol 25% at −80 °C, and considered as endophytes if no growth was observed on the sterilization control plates.

2.1.3. Amplification and sequencing of the 16S rRNA coding gene

The 16S rDNA genes of the purified isolates were amplified by colony PCR with the primers forward FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG-3') and reverse FGPS1509 (5'- AAGGAGGGGATCCAGCCGCA-3') (Ponsonnet and Nesme, 1994). The PCR amplifications were performed in 25 µL reaction mixture containing 2.5 µL DreamTaq Buffer (10X), 2 µL dNTPs (2.5 mM), 1.25 µL of each primer (10 µM) and 0.2 µL DreamTaq DNA Polymerase (5 U/µL, Thermo Fisher Scientific) and 17.8 µL sterile double-distilled water. Bacterial cells were added by touching a colony with a sterile toothpick followed by soaking it directly into the mixture. The PCR cycling conditions consisted of an initial denaturation at 95 °C for 10 min, 30 cycles of denaturation, annealing, and elongation at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, respectively and a final elongation step at 72 °C for 10 min. The amplified products were visualized under UV light after gel electrophoresis (0.8% Agarose, TAE buffer 1X, 100 V for 1 hour) and staining with ethidium bromide bath (0.5 µg/mL for 15 min). The PCR products were then sent to Macrogen Korea (Seoul, Rep. of Korea) for purification and sequencing with both previous forward and reverse primers.

2.1.4. Identification and phylogenetic analysis

Sequences analyses and phylogeny were performed using the MEGA X software (Kumar et al., 2018). The sequencing profiles of forward and reverse sequences were manually corrected if needed before being merged to obtain the nearly full-length 16S rDNA gene sequence of c.a. 1450 bp. The sequences of the eighty non redundant bacterial endophyte described in the present study, were deposited to the GenBank database under the accession numbers MT126513 to MT126592. The isolates were identified using the NCBI (https://www.ncbi.nlm.nih.gov/) BLAST, and reference strains sequences were retrieved from the Nucleotide Database. The sequences were aligned, the gaps were deleted and the phylogenetic trees were constructed using the Maximum Likelihood method (1000 bootstrap replications) with the Kimura 2-parameter model (Kimura, 1980).

2.2. Plant Growth Promoting (PGP) and Biocontrol (BC) activities screenings

Bacterial endophytes were grown from frozen glycerol stock on TSA medium at 28 °C for 24 h to 48 h in order to check the purity. Then, a single bacterial colony was inoculated with a 1 µL loop into 25 mL of Tryptic Soy Broth (TSB) medium (Merck) that were further incubated at 28 °C on a rotary shaker at 150 rpm until reaching an OD_{600nm} of 1. The time of incubation varied from for 24 h to 72 h depending of bacterial isolate. The bacterial cells were harvested by centrifugation at 2500 *g* for 10 min and resuspended in sterile water at an optic OD_{600nm} of 0.8.

These inocula were used for all the PGP and BC semi-quantitative assays, except for the gelatinase screening for which the bacteria were stab inoculated. Each isolate was tested in duplicate for all the screenings with sterile distilled water (SDW) as a negative control. The indolic compounds production was assessed on TSA medium supplemented with 5 mM of tryptophan according to Bric et al. (1991) and Frey-Klett et al. (2004) with the Salkowski reagent prepared according to Pilet and Chollet (1970) as reported by Glickmann and Dessaux (1995). The ability to solubilize inorganic phosphorus was assessed on the Pikovskaya medium prepared according to Pikovskaya (1948) as reported by Nautiyal (1999). The siderophore production was assessed on CAS Agar medium prepared according to Alexander and Zuberer (1991). The HCN production was assessed in TSA medium supplemented with 4.4 g per liter of glycine with the method of Frey-Klett et al. (2004) and the HCN revealing solution prepared according to Castric (1975). The lipase and esterase production were assessed in the basal medium prepared following Sierra (1957) and Kumar et al. (2012) supplemented with 1% (v/v) Tween 80 for lipase and 1% (v/v) Tween 20 for esterase. The chitinase production was assessed on chitin supplemented medium prepared according to Murthy and Bleakley (2012) containing 2.0% moist colloidal. The gelatinase production was assessed with a gelatin liquefaction stab method on gelatin nutrient medium containing 12% of gelatin (Dela Cruz and Torres, 2012). After 3 days incubation at 28 °C and 7 days for phosphate solubilization, chitinase and gelatinase production, the isolates were ranked in classes for each screening, referred to '0' for negative, '+' for low, '++' for medium and '+++' for high level of activity, respectively.

2.3. Direct antifungal activity on Fusarium oxysporum

The *Fusarium oxysporum* strain was kindly provided by Dr. Xuan Hoa Nguyen (Western Highlands Agriculture and Forestry Science Institute - WASI, Buon Ma Thuot, Vietnam), and maintained on Potatoes Dextrose Agar (PDA) medium (Merck) at 28 °C. The antagonistic effect of the isolated bacterial endophytes against the fungal growth was evaluated by the dual culture method. Briefly, square plugs of 1 cm^2 were cut from the edge of the fungal colony and placed in the center of fresh PDA containing 9 cm diameter Petri dishes. Four 10 µL drops of bacterial inoculum (previously described) were inoculated in opposite directions at 3.5 cm from the center of the fungal plugin triplicate for each bacterial isolate. After 5 days of incubation at 28 °C, the radii of the fungal colony were measured in the direction of the bacterial colonies. The significance of the differences between the fungal mean radius in treatment and control were evaluated with the student test ($\alpha = 0.05$). The fungal growth inhibition rate was then calculated as the mean of the replicates with the following formula: Inhibition % = (Rc-Rt)*100/Rc where Rc and Rt are the radii of the fungal colonies in the control inoculated with SDW and the treatment inoculated with bacteria, respectively. The isolates were ranked in classes, referred to '0' for negative, '+' for low, '++'medium and '+++' for high inhibition of the fungal growth.

2.4. Direct nematicidal activity screening

2.4.1. Nematode strains, isolation, disinfection, and maintenance

The two nematodes species used in this study were isolated from *C. canephora* roots in Buon Ma Thuot (Dak Lak province) and were maintained on carrot discs modified following Moody et al. (1973). The *Radopholus duriophilus* strain was kindly provided by Dr. Xuan Hoa Nguyen (WASI). The *Pratylenchus coffeae* strain was isolated according to the protocol of Hooper et al. (2005). Briefly, coffee roots were rinsed carefully with tap water, cut into 2 cm fragment, and macerated in a kitchen blender. The roots were then incubated in a modified Baermann funnel (Whitehead and Hemming, 1965) to retrieve active nematodes. Extracted nematodes were first rinsed with SDW using a 20 µm sieve and then disinfected in a glass test tube as follows. The volume was reduced to 100 µL, and 5 mL of antibiotic solution (streptomycin 0.8% (w/v) and gentamicin 0.8%(w/v)) were added, and the nematodes were incubated for 1 h at room temperature. This step was repeated twice. Finally, the volume was reduced to 100 µL, and the nematodes were resuspended in SDW before being inoculated on sterile carrot discs. After two months of incubation at 28 °C, approximately ten thousand nematodes were harvested from each carrot disc.

2.4.2. Nematicidal activity on Radopholus duriophilus and Pratylenchus coffeae

The potential nematicidal activity of the isolates was evaluated with a preliminary screening involving direct confrontations. The bacterial inocula were prepared as previously described with an additional step where the cells were washed with SDW after centrifugation at 2500*g* for 10 min in order to remove the culture medium. For each treatment, five replicates were used in 24 wells cell culture plates (SPL Life Sciences Co.,

Ltd.) with 100 nematodes (*R. duriophilus*) and the bacteria at a final OD_{600nm} of 0.8 or SDW as a control in a total volume of 1 mL. After 48 h of incubation at 28 °C, the nematodes were transferred in 1 mL on a counting cell (Sedgewick Rafter Cell, Pyser Optics, Ltd.). All the nematodes were counted and considered as dead based on the shape and the absence of mobility. The mortality relative risk (RR, equivalent to the proportion of dead nematodes in the treatments divided by the proportion of dead nematodes in controls) was then obtained after statistical analyses.

For the assessment of the lethal concentrations (LC), the bacterial inocula were diluted at eight different concentrations (OD_{600nm} 1, 0.875, 0.75, 0.625, 0.5, 0.375, 0.25 and 0.125). Three replicates for each bacterial concentration were used in 24 wells cell culture plates (SPL) with 100 nematodes (*R. duriophilus*) and SDW as a control in a total volume of 1 mL. After 24 h of incubation at 28 °C, all the alive and dead nematodes were counted. The lethal concentrations (LC_{50}, LC_{90}, and LC_{95}) were calculated after statistical analyses. The most efficient isolates were then tested in the same conditions on the second coffee parasitic nematode species (*P. coffeae*).

2.5. Statistical analyses

All the statistical analyses were conducted on the R software (R Core Team, 2019). To analyze the distribution of the bacterial genera across the different samples, a Venn diagram was constructed with the package "VennDiagram" (Chen, 2018). Multivariate analyses were conducted on the data in order to decipher the correlations between the different variables measured. The correlation circles from the Principal Component Analyses (PCA) were obtained with the package "psy" (Falissard, 2012). The correlograms were obtained with the package "corrplot" (Wei and Viliam, 2017), and the significance of the correlations was assessed with the package "Hmisc" (Harrell and Dupont, 2019). The data from the first nematicidal activity assay on *R. duriophilus* were subjected to binomial relative risk regressions using a generalized linear model (GLM) and the Log-link function with the package "logbin" (Donoghoe and Marschner, 2018) in order to calculate the relative mortality. The data from the assessment of the lethal concentrations were subjected to (quasi)-binomial regressions using generalized linear models (GLM) with different link functions (Cauchit or Complementary Log-Log or Logit), and the lethal concentrations were obtained with the package "MASS" (Venables and Ripley, 2002).

3. Results

3.1. Coffee bacterial endophytes isolation and identification

The isolates were first selected on the base of the colony morphology (shape and color). A total of one hundred and forty culturable bacterial endophytes were isolated from coffee samples (roots and seeds) collected in Bao Loc and Buon Ma Thuot. In Bao Loc, we obtained twenty-eight and fifty-three isolates from roots of *C. liberica* and *C. canephora*, respectively. In Buon Ma Thuot, forty-six isolates from roots and thirteen from the seeds of *C. canephora* were recovered. For all these isolates, the 16S rDNA was amplified, sequenced and compared to the NCBI database. Redundant isolates were identified based on 16S rDNA sequences comparison, the redundancy was confirmed functionally with the PGP/BC activities. Finally, sixty redundant isolates were removed (Fig. 1). The eighty remaining isolates were distributed in three bacterial phyla: Actinobacteria (30), Firmicutes (28), and Proteobacteria (22) (Fig. 2A). Thirty distinct genera were identified. The Actinobacteria phylum was the most diversified with fourteen different genera and *Nocardia* as the most represented genus (Fig. 2B), followed by Proteobacteria with twelve different genera and *Burkholderia* as the predominant genus (Fig. 2C) and the less diversified is the Firmicutes phylum with four different genera with *Bacillus* as dominant genus (Fig. 2D).

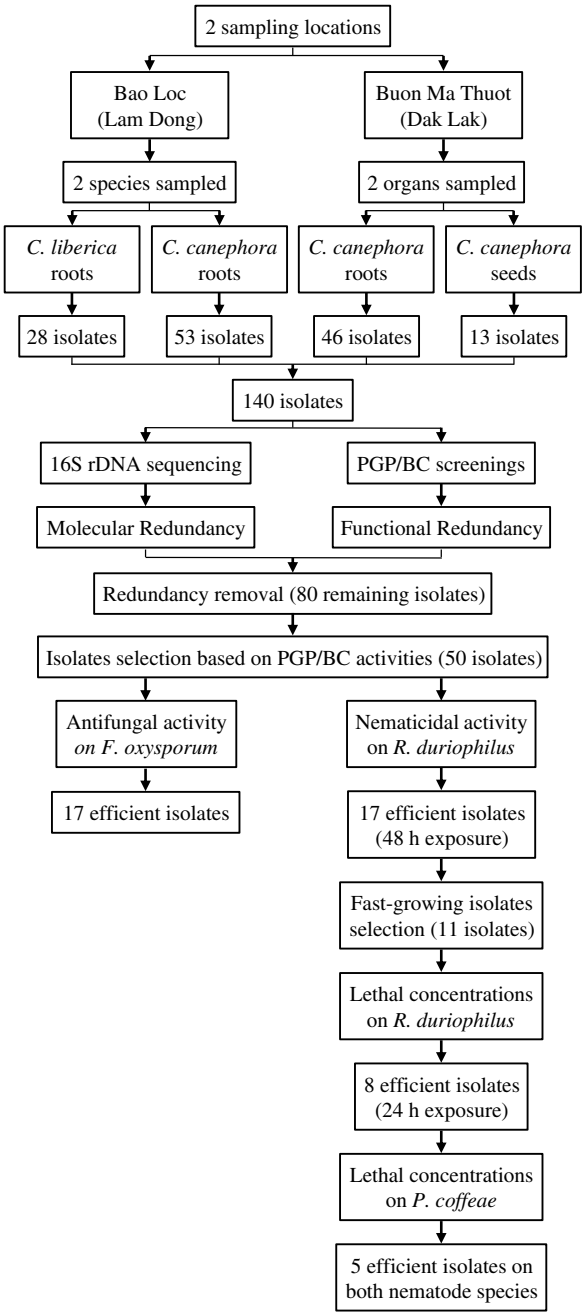

Fig. 1. Workflow diagram of the study describing the experiments performed from the sampling to the functional characterization.

Focusing on the distribution of the thirty bacterial genera across the samples, the *Bacillus* was the only genus found in all the samples, five genera (*Bacillus, Kocuria, Mycolicibacterium, Nocardia and* Pseudomonas) were shared between the two sampling locations (Bao Loc and Buon Ma Thuot), three genera (*Bacillus*, *Burkholderia* and *Nocardia*) were shared between the two *Coffea* species (*C. canephora* and *C. liberica*) and two genera (*Bacillus* and *Kocuria*) were shared between the two organs (roots and seeds). Assessing the distribution within the sampling locations, three genera (*Bacillus, Burkholderia* and *Nocardia*) were shared between *C. canephora* and *C. liberica* roots in Bao Loc while only one genus (*Bacillus*) was shared between *C. canephora* roots and seeds in Buon Ma Thuot. However, twenty-four of the thirty genera were not shared between the different samples. Indeed, the following genera were isolated exclusively from their respective samples: *Luteibacter*, *Rhizobium*, *Sphingobium*, *Sphingomonas* and *Staphylococcus* from *C. canephora* roots

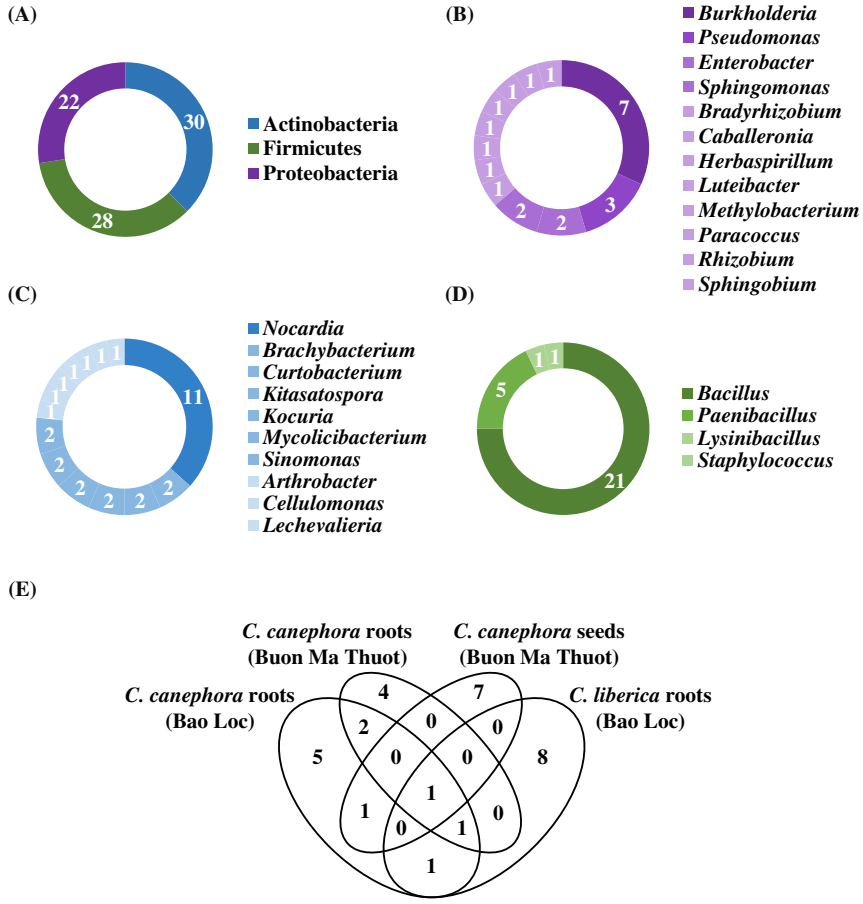

Fig. 2. Distribution of the isolates (A), Number of isolates by phyla. (B), Number of isolates by genera in the Actinobacteria phylum. (C), Number of isolates by genera in the Proteobacteria phylum. (D), Number of isolates by genera in the Firmicute phylum. (E), Venn diagram showing the bacterial genera distribution across the samples.

in Bao Loc; *Bradyrhizobium*, *Kitasatospora*, *Lechevalieria* and *Streptomyces* from *C. canephora* roots in Buon Ma Thuot; *Brachybacterium*, *Caballeronia*, *Curtobacterium*, *Methylobacterium*, *Mycobacterium*, *Nakamurella* and *Paracoccus* from *C. canephora* seeds in Buon Ma Thuot; as well as *Arthrobacter*, *Cellulomonas*, *Enterobacter*, *Herbaspirillum*, *Leifsonia*, *Lysinibacillus*, *Paenibacillus* and *Sinomonas* from *C. liberica* roots in Bao Loc (Fig. 2E). The bacterial genera in each partition of the Venn diagram are available (Supplementary Table S1).

Finally, the 16S rDNA sequences of the eighty isolates were used to construct two phylogenetic trees including or not eighty-two reference strains (Supplementary Fig. S2, Supplementary Datasets S1-2) and the most closely related species were assigned (Supplementary Table S2).

3.2. Plant growth-promoting and biocontrol activities screenings

The one hundred and forty isolated bacterial endophytes were characterized for several activities known for their involvement in plant growth promotion (PGP: indolic compounds production, phosphate solubilization, siderophore production), and/or in pathogens biocontrol (BC: esterase, lipase, gelatinase, chitinase activities, HCN production). The results were used to confirm the molecular redundancy (16S rDNA sequences) by using functional tests and were analyzed in detail for the eighty remaining isolates (Supplementary Table S3). Among the PGP activities, thirty isolates were able to solubilize the phosphate, followed by twenty-five indolic compounds producing isolates. The siderophores, which are considered as both PGP and BC compounds, were produced by twenty-three isolates. Concerning the BC activities, esterase was the most commonly produced enzyme with thirty-four isolates, followed by lipase, gelatinase, and chitinase with twenty-five, fourteen, and eleven isolates, respectively. Regarding the production of HCN, only one isolate was able to produce this gaseous compound (Table 1A). By analyzing the number of activities per isolate, only twelve isolates were found to have none, and twenty-seven isolates exhibited only one of the activities sought. The other isolates had two to six cumulated activities and represented more than 50% of the isolates (Table 1B). Finally, fifty isolates displaying at minima a medium activity for one of the screenings or several cumulated activities were selected for further analyzed of their direct antifungal and nematicidal abilities on *F. oxysporum* and

Table 1
Overview of the PGP/BC activities (A) Number of isolates by activity; 'Est', Esterase production; 'Pho', Phosphate solubilization; 'Ind', Indolic compounds production; 'Lip', Lipase production; 'Sid', Siderophores production; 'Gel', Gelatinase production; 'Chi', Chitinase production; 'Hcn', Hydrogen cyanide production. (B) Number of isolates by number of cumulated activities.

(A)

	Number of isolates by activity							
	Est	Pho	Ind	Lip	Sid	Gel	Chi	Hcn
Number	34	30	25	25	23	14	11	1
% of total	42.5	37.5	31.25	31.25	28.75	17.5	13.75	1.25

(B)

	Number of isolates with multiple activities						
	0	1	2	3	4	5	6
Number	12	27	13	13	8	3	4
% of total	15	33.75	16.25	16.25	10	3.75	5

R. duriophilus, respectively (Supplementary Table S3).

3.3. Direct antifungal activity on F. oxysporum screening

Dual culture method was used to identify the bacterial isolates that were able to inhibit the growth of *F. oxysporum*. The fifty isolates selected after the screening of PGP/BC activities were tested and seventeen were significantly able to inhibit the growth of the plant pathogenic fungus (Table 2). Among the efficient isolates, the Proteobacteria phylum was the most represented with eight isolates belonging to the *Burkholderia, Enterobacter, Luteibacter,* and *Pseudomonas* genera. Six isolates belonged to the Actinobacteria phylum and the genera *Brachybacterium, Curtobacterium, Kitasatospora*, and *Streptomyces*. The less represented phylum was the Firmicutes with three isolates, all from the *Bacillus* genus. The three most efficient isolates displayed an inhibition rate higher than 40% (CLBLR18: *Burkholderia* sp., CCBLR24: *Bacillus* sp. and CCBMTR6: *Streptomyces* sp.). Four isolates exhibited a medium biocontrol activity between 26% and 31% (CCBLR23: *Bacillus* sp., CLBLR6: *Enterobacter* sp., CLBLR13: *Burkholderia* sp. closely related to the *B. seminalis* and CCBLR25 closely related to *B cenocepacia*). The remaining positive isolates displayed antifungal activities against *F. oxysporum* ranging from 8% and 23% of inhibition.

3.4. Direct nematicidal activity screening

Direct confrontations between the fifty selected isolates and nematodes were used to identify a nematicidal effect on *R. duriophilus*. The mortality relative risk (RR) was calculated, and seventeen isolates presented a value higher than 2, corresponding to a mortality prevalence at least two times higher in the treatments compared to the controls (Table 3). Among these isolates, the most represented phylum was the Firmicutes with nine isolates exclusively from the *Bacillus* genus, followed by the Proteobacteria phylum with five isolates belonging to the *Enterobacter*, *Herbaspirillum*, *Methylobacterium*, *Paracoccus,* and *Pseudomonas* genera. The less represented phylum was the Actinobacteria with three isolates belonging to the *Arthrobacter*, *Lechevalieria,* and *Streptomyces* genera. The most efficient isolates displayed an RR value comprised between 13 and 18 (CCBMTR1: *Bacillus* sp., CCBLR2: *Bacillus* sp., CCBMTS9: *Methylobacterium* sp. and CLBLR16: *Paenibacillus* sp.). One isolate exhibited a medium RR value of 11.12 (CLBLR3: *Bacillus* sp.) The twelve remaining isolates had an RR value between 2 and 6.

A lethal concentrations (LC) assessment was conducted on the most efficient bacterial isolates Among the seventeen isolates presenting a RR value higher than 2, only fast-growing were retained to potentially select the most competitive ones (Table 3). Six slow-growing isolates were not retained: CCBMTS9, *Methylobacterium* sp., CLBLR16 *Paenibacillus* sp., CLBLR3 *Bacillus* sp., *CCBMTR13 Lechevalieria* sp., CCBMTS12 *Paracoccus* sp., CCBMTR6 *Streptomyces* sp. The eleven remaining isolates were first tested on *R. duriophilus*. When they induced at least 50% of mortality (LC_{50}) on *R. duriophilus* in the concentration range, they were then tested on *P. coffeae*. As the mortality is a binary variable, (quasi)-binomial regressions were used to fit the sigmoidal curve of the mortality in function of the bacterial OD_{600nm} (Supplementary Figures S2-3), and the lethal concentrations were calculated (Table 4). Three isolates were not efficient at 24 h on *R. duriophilus* (CCBLR2, *Bacillus* sp., *CCBLR22, Pseudomonas* sp. and CLBLR6, *Enterobacter* sp.). The eight remaining isolates were all able to lead to 100% of *R. duriophilus* mortality in the range of concentrations tested except the isolate CLBLR5, *Herbaspirillum* sp., which led to only 70% of mortality. The $OD_{600\ nm}$ values were equivalent to a range between 10^4 and 10^9 CFU/mL depending on the isolate. Three isolates were efficient only on *R. duriophilus* (CCBMTR1, *Bacillus* sp., CLBLR5, *Herbaspirillum* sp. and CLBLR12, *Arthrobacter* sp.) while the five remaining isolates all belonging to the *Bacillus* genus were efficient on both nematode species (CCBLR15, CCBLR14, CCBLR1, CCBLR13, and CCBMTR4). On *R. duriophilus,* the smallest LC_{50}, $LC_{90,}$ and LC_{95} (0.18 ± 0.01, 0.35 ± 0.01 and 0.53 ± 0.03 respectively) were obtained with the isolate CCBLR13. On *P. coffeae*, the smallest LC_{50} (0.35 ± 0.01) was displayed by the isolate CCBMTR4, while the smallest LC_{90} and LC_{95} (0.47 ± 0.01 and 0.48 ± 0.01, respectively) were exhibited by the isolate CCBLR14.

3.5. Multivariate analysis

By focusing on the PGP/BC screenings, the multivariate analysis illustrated that most of the activities seemed to be positively correlated except for the indolic compounds production that tended to be negatively correlated with the others (Fig. 3A). Regarding the antifungal activity, several activities were positively correlated with the *F. oxysporum* growth inhibition. The highest significant correlation was with the siderophores production ($r = 0.33$), followed by gelatinase production ($r = 0.32$) and phosphate solubilization ($r = 0.29$). Finally, the lipase production tended to be positively correlated, and the production of the indolic compound tended to be negatively correlated with the *F. oxysporum* growth inhibition (Fig. 3B). Concerning the nematocidal activity, the multivariate analysis did not identify any significant correlation between the PGP/BC activities and the nematocidal activity

Table 2
Antifungal activity on *F. oxysporum* with the last column 'Foxy' equivalent to the percentage of fungal growth inhibition with standard error. The genera, closely related species and PGP/BC activities of the seventeen positive isolates are indicated. 'Ind', Indolic compounds production; 'Pho', Phosphate solubilization; 'Sid', Siderophores production; 'Hcn', Hydrogen cyanide production; 'Gel', Gelatinase production; 'Chi', Chitinase production; 'Lip', Lipase production; 'Est', Esterase production.

Isolate	*Genus*	*Closely related species*	Ind	Pho	Sid	Hcn	Gel	Chi	Lip	Est	Foxy (%±SE)
CLBLR18	*Burkholderia*	*cenocepacia*	-	+++	+++	-	++	+	+++	++	49.77 ± 0.08
CCBLR24	*Bacillus*	*subtilis*	-	-	-	-	+++	-	+	++	40.77 ± 0.06
CCBMTR6	*Streptomyces*	*mobaraensis*	-	-	-	-	-	+	-	+	40.76 ± 0.04
CCBLR23	*Bacillus*	*subtilis*	-	-	+++	-	+++	-	+	-	30.52 ± 0.01
CLBLR6	*Enterobacter*	*cancerogenus*	+++	+++	+	-	-	-	-	++	29.17 ± 0.07
CLBLR13	*Burkholderia*	*seminalis*	-	+	+++	-	-	-	+++	++	26.99 ± 0.01
CCBLR25	*Burkholderia*	*cenocepacia*	-	+	+++	-	-	-	+++	+++	26.50 ± 0.01
CCBLR22	*Pseudomonas*	*nitroreducens*	-	-	+++	-	-	-	-	-	22.18 ± 0.04
CLBLR8	*Bacillus*	*altitudinis*	-	-	-	-	+++	-	-	++	18.43 ± 0.15
CCBMTS5	*Curtobacterium*	*oceanosedimentum*	-	+	-	-	-	+	-	-	17.68 ± 0.06
CCBMTR3	*Kitasatospora*	*phosalacinea*	-	-	-	-	-	+++	++	+++	13.05 ± 0.05
CLBLR7	*Enterobacter*	*asburiae*	++	++	+	-	-	-	-	-	12.65 ± 0.06
CCBMTS7	*Curtobacterium*	*citreum*	-	+	-	-	-	-	+++	+	12.64 ± 0.03
CCBLR21	*Pseudomonas*	*putida*	-	+++	++	+	+++	-	+++	++	11.30 ± 0.04
CCBMTS8	*Brachybacterium*	*squillarum*	+	+	-	-	++	-	-	-	10.26 ± 0.09
CCBMTR5	*Kitasatospora*	*arboriphila*	-	-	-	-	-	++	-	-	9.00 ± 0.04
CCBLR5	*Luteibacter*	*yeojuensis*	+++	++	+	-	++	-	+	+	8.62 ± 0.02

Table 3
Nematicidal activity on *R. duriophilus* with the last column 'Rado' equivalent to the mortality relative risk (RR) after 48 h at $OD_{600nm} = 0.8$ compared to the negative control with standard error. The genera, closely related species and PGP/BC activities of the seventeen efficient isolates are indicated. The eleven isolates in bold were selected for the assessment of the lethal concentrations. 'Ind', Indolic compounds production; 'Pho', Phosphate solubilization; 'Sid', Siderophores production; 'Hcn', Hydrogen cyanide production; 'Gel', Gelatinase production; 'Chi', Chitinase production; 'Lip', Lipase production; 'Est', Esterase production.

Isolate	*Genus*	*Closely related species*	Ind	Pho	Sid	Hcn	Gel	Chi	Lip	Est	Rado (RR ± SE)
CCBMTR1	***Bacillus***	***timonensis***	++	-	-	-	-	-	-	++	**17.95 ± 1.17**
CCBLR2	***Bacillus***	***timonensis***	++	-	-	-	-	-	-	-	**16.52 ± 1.17**
CCBMTS9	*Methylobacterium*	*tardum*	++	-	-	-	-	-	-	-	14.22 ± 1.17
CLBLR16	*Paenibacillus*	*cellulosilyticus*	-	-	-	-	-	-	++	-	13.86 ± 1.17
CLBLR3	*Bacillus*	*wuyishanensis*	+	-	-	-	-	-	+	-	11.12 ± 1.18
CCBLR15	***Bacillus***	***cereus sensu lato***	-	+	+	-	+	+	-	+	**5.56 ± 1.09**
CCBLR14	***Bacillus***	***mycoides***	-	+	-	-	++	-	-	+	**5.52 ± 1.09**
CCBLR1	***Bacillus***	***cereus sensu lato***	-	+	-	-	+	+	+	+	**5.36 ± 1.09**
CCBLR13	***Bacillus***	***cereus sensu lato***	-	+	-	-	-	+	+	++	**5.23 ± 1.09**
CCBMTR13	*Lechevalieria*	*aerocolonigenes*	-	-	++	-	+++	+++	+	+++	5.03 ± 1.09
CCBMTS12	*Paracoccus*	*sanguinis*	+++	-	-	-	-	-	-	-	3.19 ± 1.08
CLBLR5	***Herbaspirillum***	***frisingense***	-	-	+++	-	-	-	-	-	**2.75 ± 1.06**
CCBLR22	***Pseudomonas***	***nitroreducens***	-	-	+++	-	-	-	-	-	**2.51 ± 1.06**
CCBMTR6	*Streptomyces*	*mobaraensis*	-	-	-	-	-	+	-	+	2.45 ± 1.21
CCBMTR4	***Bacillus***	***cereus sensu lato***	-	+	+	-	+	+	+	++	**2.38 ± 1.06**
CLBLR12	***Arthrobacter***	***phenanthrenivorans***	++	-	-	-	-	-	-	-	**2.36 ± 1.06**
CLBLR6	***Enterobacter***	***cancerogenus***	+++	+++	+	-	-	-	-	++	**2.32 ± 1.05**

Table 4
Assessment of the lethal concentrations on *R. duriophilus* and *P. coffeae* after 24 h with the columns 'LC_{50}', 'LC_{90}' and 'LC_{95}' equivalent to the OD_{600nm} values corresponding to the lethal concentrations with 50%, 90% and 95% of nematodes mortality with standard errors. The genera, closely related species of the isolates are indicated. The isolates in bold were efficient on both nematode species. 'Rado', *R. duriophilus* mortality relative risk (RR) after 48 h at $OD_{600nm} = 0.8$; 'NE', not-efficient at 24 h; 'NA', not-available; 'CFU/mL', colony forming unit per mL; '*', concentration obtained with the fitted model.

				Radopholus duriophilus lethal concentrations (OD_{600nm}, 24 h)			*Pratylenchus coffeae* lethal concentrations (OD_{600nm}, 24 h)			
Isolate	*Genus*	*Closely related species*	Rado (RR ± SE)	LC50 ± SE	LC90 ± SE	LC95 ± SE	LC50 ± SE	LC90 ± SE	LC95 ± SE	CFU/mL
CCBMTR1	*Bacillus*	*timonensis*	17.95 ± 1.17	0.53 ± 0.02	0.78 ± 0.02	0.83 ± 0.02	NE	NE	NE	10^4-10^5
CCBLR2	*Bacillus*	*timonensis*	16.52 ± 1.17	NE	NE	NE	NA	NA	NA	10^5-10^6
CCBLR15	***Bacillus***	***cereus sensu lato***	**5.56 ± 1.09**	**0.20 ± 0.01**	**0.36 ± 0.02**	**0.54 ± 0.03**	**0.40 ± 0.01**	**0.52 ± 0.01**	**0.56 ± 0.02**	**10^6-10^7**
CCBLR14	***Bacillus***	***mycoides***	**5.52 ± 1.09**	**0.31 ± 0.01**	**0.48 ± 0.01**	**0.54 ± 0.01**	**0.39 ± 0.00**	**0.47 ± 0.01**	**0.48 ± 0.01**	**10^6-10^7**
CCBLR1	***Bacillus***	***cereus sensu lato***	**5.36 ± 1.09**	**0.61 ± 0.02**	**0.83 ± 0.02**	**0.88 ± 0.02**	**0.55 ± 0.01**	**0.72 ± 0.01**	**0.78 ± 0.02**	**10^6-10^7**
CCBLR13	***Bacillus***	***cereus sensu lato***	**5.23 ± 1.09**	**0.18 ± 0.01**	**0.35 ± 0.01**	**0.53 ± 0.03**	**0.50 ± 0.01**	**0.63 ± 0.02**	**0.66 ± 0.02**	**10^6-10^7**
CLBLR5	*Herbaspirillum*	*frisingense*	2.75 ± 1.06	0.83 ± 0.02	1.38 ± 0.07*	1.96 ± 0.14*	NE	NE	NE	10^7-10^8
CCBLR22	*Pseudomonas*	*nitroreducens*	2.51 ± 1.06	NE	NE	NE	NA	NA	NA	10^7-10^8
CCBMTR4	***Bacillus***	***cereus sensu lato***	**2.38 ± 1.06**	**0.54 ± 0.01**	**0.75 ± 0.03**	**0.97 ± 0.05**	**0.35 ± 0.01**	**0.49 ± 0.01**	**0.53 ± 0.01**	**10^6-10^7**
CLBLR12	*Arthrobacter*	*phenanthrenivorans*	2.36 ± 1.06	0.23 ± 0.02	0.48 ± 0.03	0.55 ± 0.04	NE	NE	NE	10^8-10^9
CLBLR6	*Enterobacter*	*cancerogenus*	2.32 ± 1.05	NE	NE	NE	NA	NA	NA	10^7-10^8

on *R. duriophilus*. All the PGP/BC capacities seemed to be negatively correlated except a weak positive correlation with the indolic compound production (r = 0.17) (Fig. 3B).

4. Discussion

Coffee bacterial endophytes gained substantial attention since they were first reported in roots, stems (Jimenez-Salgado et al., 1997), cherries (Sakiyama et al., 2001), and from other coffee tissues (e.g leaves, cherries crown, peduncle, pulp and seeds) (Vega et al., 2005). In the present study, *C. canephora* roots samples were collected in nematode infected fields in the two main Vietnamese coffee producing regions with the primary objective to isolate endophytic bacterial antagonists of soilborne diseases responsible for the replanting failure issue in Vietnam including nematodes which are sometimes in association with fungal pathogens (Chapman, 2014; Loang, 2002). Indeed, it has already been demonstrated that bacterial endophytes isolated from coffee root in Vietnam were able to reduce eggs hatching and to display a nematicidal activity toward the root knot nematode *M. incognita*. Furthermore, *C. liberica* roots were also sampled as it is commonly used as coffee rootstock and is believed to be more resistant to nematodes (Bally and Reydon, 1931; Obregon and Rafael, 1936; Wiryadiputra et al., 1994 in Souza, 2008). Finally, as the second location was sampled during the harvest period, roots but also cherries were collected in order to give an insight in the shared bacterial endophytes between roots and seeds that could potentially be vertically transmitted over generations (Frank et al., 2017; Truyens et al., 2015).

To our knowledge, the present study is the first description of bacterial endophytes from *C. liberica*. In this work, eighty coffee bacterial endophytes were characterized: forty-six and twenty-two were recovered from the roots of *C. canephora* and *C. liberica*, respectively, and twelve isolates were obtained from *C. canephora* seeds. All bacterial endophytes isolated were distributed in the Actinobacteria, Firmicutes, and Proteobacteria phyla. These isolates were representative of the already known culturable endophytes diversity described from several parts of the coffee trees including cherries, leaves, branches, stems and

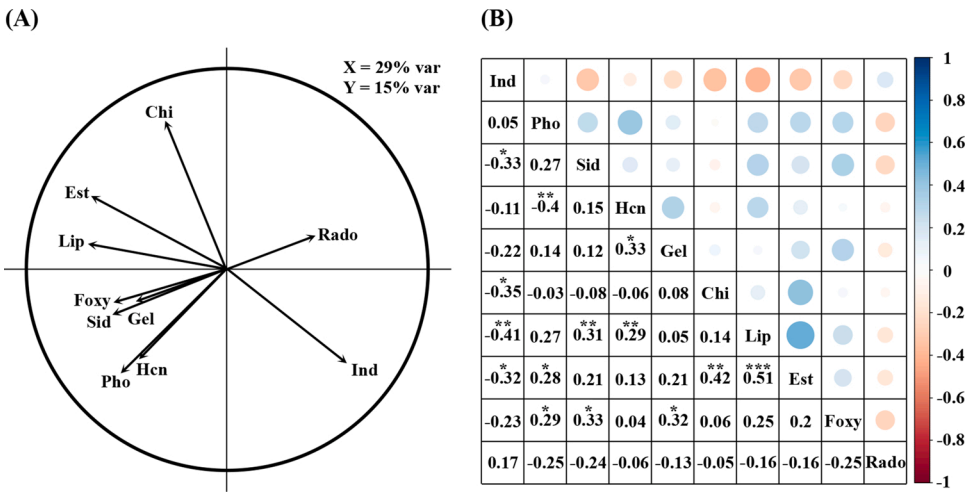

Fig. 3. Multivariate analysis of the PGP/BC activities, antifungal activity on *F. oxysporum* and nematicidal activity on *R. duriophilus*. (A), Correlation circle, arrows represent the variables. (B), Correlogram with the pearson correlation coefficients and circles with proportional color and size. The significance of each coefficient is indicated: ***, *p*value<0.001; **, *p*value<0.01 and *, *p*value<0.05. 'Ind', Indolic compounds production; 'Pho', Phosphate solubilization; 'Sid', Siderophores production; 'Hcn', Hydrogen cyanide production; 'Gel', Gelatinase production; 'Chi', Chitinase production; 'Lip', Lipase production; 'Est', Esterase production; 'Foxy', antifungal activity on *F. oxysporum* (percentage of growth inhibition); 'Rado', nematicidal activity on *R. duriophilus* (mortality relative risk).

roots (Asyiah et al., 2018; Hoang et al., 2020; Jimenez-Salgado et al., 1997; Nair et al., 2002; Nunes and de Melo, 2006; Sakiyama et al., 2001; Shiomi et al., 2006; Teshome et al., 2017; Vaughan et al., 2015; Vega et al., 2005). However, Miguel et al. (2013) and Mekete et al. (2009), isolated endophytes belonging to the phylum Bacterioidetes from coffee cherries and roots, but the relative abundance of this bacterial phylum was very low with *Chryseobacterium* as only genus. Recently, Fulthorpe et al. (2020) described the *C. arabica* roots endophytic community with a metabarcoding approach. While the authors identified the Actinobacteria and Proteobacteria as predominant phyla, they also reported members of the Acidobacteria, Chloroflexi and Planctomycetes phyla.

Out of the thirty genera described in the present study, twelve have never been reported before in endophytic association with coffee. They included members of the genera *Brachybacterium*, *Caballeronia*, *Nakamurella* and *Paracoccus* isolated from the seeds and the genera *Kitasatospora*, *Lechevalieria*, *Leifsonia*, *Luteibacter*, *Lysinibacillus*, *Mycolicibacterium*, *Sinomonas* and *Sphingobium* isolated from the roots. Additionally, the present work provides new insights into the localization of some genera already know to be in endophytic association with coffee. Thus, endophytic genera associated with coffee cherries and seeds (Miguel et al., 2013; Oliveira et al., 2013; Vaughan et al., 2015) were recovered for the first time from the roots including members of *Rhizobium*, *Staphylococcus,* and *Sphingomonas.* Furthermore, members of the *Mycobacterium* genus already described as coffee root endophytes have never been isolated from the seeds (Teshome et al., 2017).

Regarding the distribution of the bacterial genera across the different samples, we highlighted that the *Bacillus* genus was present in all the samples, namely, *C. canephora* and *C. liberica* roots in Bao Loc as well as the *C. canephora* roots and seeds in Buon Ma Thuot. Moreover, it has already been demonstrated in various countries that members of this bacterial genus were able to colonize endophytically almost all coffee plant organs including cherries (Miguel et al., 2013; Oliveira et al., 2013), leaves (Bettiol et al., 2007; Shiomi et al., 2006; Silva et al., 2008; Vega et al., 2005), roots (Asyiah et al., 2018; Hoang et al., 2020; Mekete et al., 2009; Teshome et al., 2017; Vega et al., 2005), seeds (Vega et al., 2005) and stems (Nair et al., 2002; Shiomi et al., 2006). These findings indicate that the *Bacillus* genus members are competitive coffee colonizers able to adapt themselves to diverse environmental conditions. Despite the fact that other genera were also shared between samples, most of the genera described in the present study were recovered only in their respective samples highlighting that different environmental conditions, plant genotypes and plant organs had an impact on the bacterial endophyte communities. Indeed, it has been recently demonstrated through a metabarcoding analysis that coffee bacterial endophyte communities were significantly influenced by environmental parameters across a climatic gradient (Fulthorpe et al., 2020). Moreover, the impact of plant genotypes and organs studied on endophyte communities has already been demonstrated in another perennial crop such as olive tree (Mina et al., 2020). Finally, only two (*Bacillus* and *Kocuria*) of the nine genera recovered as seed endophytes were shared with the roots. Thus, no clear evidence of a potential vertical transmission of the root endophytes through the seeds was highlighted by our results.

In the present study, we limited the bacterial identifications at the genus level. Indeed, care should be taken with the 16S rDNA gene-based identifications. Despite that standard cutoffs of sequences similarity were established to define if two strains are belonging to the same genus or species (Stackebrandt and Ebers, 2006; Stackebrandt and Goebel, 1994), it has been demonstrated that the cut-off for the species cannot be applied to all genera (Rossi-Tamisier et al., 2015). Therefore, further characterizations such as DNA-DNA hybridization or Multi Locus Sequence Typing (MLST) are needed for an accurate species identification (Janda and Abbott, 2007; Larsen et al., 2012). To the best of our knowledge, the present work provides the most diversified picture of the culturable bacterial endophytes in coffee.

The endophytic isolates were screened for several plant growth-promoting "PGP" (Olanrewaju et al., 2017) and biocontrol "BC" activities (Compant et al., 2005). These screenings aimed to select the potentially beneficial isolates, to confirm the molecular redundancy identified with the 16S rDNA sequences in order to reduce the number of isolates to be tested on the nematodes and *F. oxysporum*.

Among the eighty non-redundant isolates, 31.25% of the isolates were able to synthesize indolic compounds known to be involved in plant growth promotion, sometimes pathogenesis, and endophytic colonization (Hardoim et al., 2015; Spaepen et al., 2007). Although the plant growth-promoting effect of bacterial indolic compounds was demonstrated in several annual crop plants (Harikrishnan et al., 2014; Myo et al., 2019), this effect still needs to be demonstrated on a perennial crop like coffee. Regarding the phosphate solubilization, 37.5% of the isolates were able to display this capacity which is an important mechanism involved in plant growth promotion by micro-organisms (Rodríguez and Fraga, 1999; Zaidi et al., 2009). Phosphate solubilizing bacteria have been shown to have a positive effect on the germination of seeds and growth of *C. arabica* and *C. canephora* in greenhouse experiments (Baon et al., 2012; Kunwar et al., 2018). We also reported that 28.75% of the isolates synthetized siderophores. The roles of these compounds in plant growth promotion are well-known either by providing a supplementary source of iron to the plant, by competing with phytopathogen for iron or by stimulating the plant defenses (Aznar and Dellagi, 2015; Crowley, 2006; Pahari et al., 2017).

The isolates described in our study were also characterized for several enzymatic activities involved in plant pathogens and pests

biocontrol. It was demonstrated that 42.5%, 31.25%, 17.5%, and 13.75% of them were able to synthesize esterase, lipase, gelatinase, and chitinase, respectively. One the one hand, esterases have already been shown to be involved in the detoxification of some virulence factor as the albicidin produced by the bacterial pathogen *Xanthomonas albilineans*, the causative agent of sugar cane leaf scald disease (Zhang and Birch, 1997, 1996), and the brefeldin A produced by the fungal pathogen *Alternaria carthami*, the causal agent of the blight disease in safflower (Kneusel et al., 1994). Furthermore, the implication of esterases in the control of bacterial disease caused by *Ralstonia solanacearum* through the degradation of its quorum sensing signal molecule has also been reported (Achari and Ramesh, 2018, 2015; Shinohara et al., 2007).

On the other hand, it has already been demonstrated that lipases, gelatinases, and chitinases were often involved in the biocontrol of plant pestspathogens such as insects, fungi, and nematodes (Castaneda-Alvarez and Aballay, 2016; Geng et al., 2016; Millew and Sands, 1977; Paiva et al., 2013; Supakdamrongkul et al., 2010; Swiontek Brzezinska et al., 2014; Tian et al., 2007; Veliz et al., 2017; Zheng et al., 2016). Finally, only one *Pseudomonas* sp. isolate was positive for the HCN production. This volatile compound has already been demonstrated to control the development *in vitro* and *in planta* of the fungal pathogen *Sclerotinia sclerotiorum* (Nandi et al., 2017), the bacterial pathogen *Agrobacterium tumefaciens* and the parasitic nematode *M. incognita* (Abd El-Rahman et al., 2019). In the literature, several bacteria from the rhizosphere were described as HCN producers (Ahmad et al., 2008; Ghodsalavi et al., 2013). However, HCN production can be deleterious for plant growth (Blom et al., 2011). Therefore, the low number of HCN-producing bacterial strains in the present study raises the question of their potential counterselection as coffee endophytes. According to the results of these screenings, fifty isolates likely to have a pathogen biocontrol effect combined with potential plant growth-promoting effect were selected for direct confrontations with *F. oxysporum* and the nematodes.

Bacterial endophytes are prime candidates for the biocontrol of plant fungal pathogens including *F. oxysporum* through the production of antifungal compounds, enzymatic degradation of the cell wall, and competition for the same ecological niches (Berg and Hallmann, 2006). *Fusarium* spp., including *F. oxysporum*, are the causative agent of the *Fusarium* wilt, also known as tracheomycosis, which affects *C. arabica, C. canephora*, and *C. liberica* species (Serani et al., 2007; Waller et al., 2007). Moreover, *F. oxysporum* has been associated with nematodes in some disease complexes (Bertrand et al., 2000; Negron and Acosta, 1989). Among the fifty isolates selected after the PGP/BC screenings, seventeen were able to efficiently inhibit the growth of *F. oxysporum*. The most efficient isolates were belonging to genera containing species already recognized as antagonists of *F. oxysporum* (Araújo et al., 2017; Jangir et al., 2018). Recently, Hoang et al. (2019) already showed that several endophytic *Streptomyces* isolated from *C. canephora* in Vietnam were able to inhibit the growth of *F. oxysporum.*

The multivariate analysis highlighted significant correlations between the *F. oxysporum* inhibition and the production of siderophores, gelatinase and the phosphate solubilization. Araújo et al. (2017) also described that the inhibition of *F. oxysporum* by an isolate of *Burkholderia seminalis* was mainly attributed to the production of siderophores. Moreover, the siderophores implication in the *F. oxysporum* biocontrol was demonstrated since the 1980's (Kloepper et al., 1980). Surprisingly, gelatinase activity was found to be correlated with the antifungal capacity of our isolates despite the fact that the implication of gelatinases in the control of fungal pathogens has never been demonstrated. Gelatinases represent a particular class of proteases which degrade the denatured collagen (gelatin). However, it is often hard to make the distinction between bacterial collagenases, gelatinases and other proteases as several bacterial proteases are able to hydrolyze single stranded collagen and gelatin (Duarte et al., 2016; Juarez and Stinson, 1999; Uesugi et al., 2008; Watanabe, 2004). Contrastingly, it has already been demonstrated that bacterial proteases were involved in antagonistic relationship against fungal pathogens including *Fusarium solani* (Yen et al., 2006), *Colletotrichum coccodes* (Palaniyandi et al., 2013), *F. udum*, *Alternaria* sp. and *Rhizoctonia* sp. (Singh and Chhatpar, 2011), as well as the fungus-like pathogen *Pythium ultimum* (Dunne et al., 2000, 1997). According these results, we hypothesized that the effect we observed could be rather attributed to some proteases than gelatinases. Finally, Ravindra Naik et al. (2008) and Jahangir et al. (2016) emphasized the potential of phosphate solubilizing bacteria in the biocontrol of phytopathogenic fungi, including *F. oxysporum.* The antifungal effect of organic acids has been reported (Guimarães et al., 2018; Hassan et al., 2015) while their production is a known mechanism of the bacterial phosphate solubilization (Chen et al., 2016; Li et al., 2018).

Focusing on nematode biocontrol, the fifty selected bacterial isolates were examined for their potential nematicidal effect on *R. duriophilus* and *P. coffeae.* Seventeen isolates exhibited a *R. duriophilus* relative mortality higher than two after 48 h and the effect was further confirmed on the second nematode species (*P. coffeae*) with five isolates leading to 100% of mortality in only 24 h. Thus, we hypothesized that the effect of the bacteria resuspended in water could be attributed either to a *de novo* secretion or the release of some compounds after the lysis of the bacterial cells.

In our study, the multivariate analysis highlighted that most of the PGP/BC activities screened tended to be negatively correlated with the nematode mortality. The indolic compound production was the only capacity positively correlated with the nematode mortality, although the correlation was not significant. However, this result is corroborated by the already reported nematicidal activity of purified indole-3-acetic acid (Bogner et al., 2017).

Altogether these results support the hypothesis that the effect could be attributed to some potentially unknown nematicidal compounds as those recently discovered by several authors (Gao et al., 2016; Geng et al., 2017; Huang et al., 2018; Luo et al., 2018; Zeng et al., 2015; Zheng et al., 2018)

In the present work, five endophytic bacteria isolates (CCBLR15, CCBLR14, CCBLR1, CCBLR13, and CCBMTR4) all belonging to the *Bacillus* genus were able to lead to 100% of mortality on both *R. duriophilus* and *P. coffeae*. The analysis of their 16S rDNA sequences revealed that these isolates shared between 98.7% and up to 100% identity with sixteen species all belonging to the *Bacillus cereus sensu lato* group including *B. albus, B. anthracis, B. cereus sensu stricto, B. gaemokensis, B. mobilis, B. mycoides, B. nitratireducens, B. pacificus, B. paramycoides, B. paranthracis, B. proteolyticus, B. pseudomycoides, B. thuringiensis, B. toyonensis, B. tropicus* and *B. wiedmannii.*

It is worth to note that this subdivision of the *Bacillus* genus contain more than eighteen closely related species including *B. thuringiensis* commonly used as biopesticide and *B. anthracis* the well-known causative agent of anthrax (Acevedo et al., 2019; Palma et al., 2014). Ash et al. (1991) demonstrated that the 16S rDNA sequences of *B. anthracis*, *B. cereus sensu stricto*, *B. mycoides*, and *B. thuringiensis* were sharing more than 99% of similarities. These results illustrated the limits of the identifications at the species level based on the 16S rDNA sequences.

The nematode control capacities of several endophytic isolates belonging to this group was already described in the literature. Aravind et al. (2010) and Tran et al. (2019) demonstrated that endophytic strains of black pepper closely related *B. cereus, B. thuringiensis, B. proteolyticus* and *B. wiedmannii* were efficient in controlling some *Meloidogyne* spp. and *Radopholus similis in vitro* and *in planta*. In another study, Hu et al. (2017) showed that a *B. cereus* strain isolated from strawberry was able to control *M. incognita in vitro* and on tomato. In addition, Mekete et al. (2009) reported the ability of coffee root endophytic *B. mycoides* to control the plant-parasitic nematode *M. incognita* on tomato by reducing the number of galls and eggs. Finally, Asyiah et al. (2018) isolated a strain closely related *to B. anthracis* from coffee roots and highlighted its capacity to reduce by 90% the penetration of *P. coffeae* in the seedlings. However, a particular attention must be taken before considering the

use of a strain belonging to the *B. cereus sensu lato* group for agronomical purpose because of the human pathogenicity of some of the members.

Finally, among these five most efficient isolates, only one (CCBLR14) has been successfully identified as *B. mycoides* based on its characteristic rhizoidal colony pattern (Franco et al., 2002). This isolate is particularly interesting as *B. mycoides* is widely recognized as a non-pathogenic member of the *cereus* group (Bargabus et al., 2002; Nakamura and Jackson, 1995)

5. Conclusion

The present study demonstrates that coffee endophytic bacteria harbor a plethora of activities, thus presenting a high potential for the development of plant-growth-promoting and biocontrol agents. For the first time, several bacterial genera were described as coffee endophytes. Three isolates belonging to the *Bacillus*, *Burkholderia*, and *Streptomyces* genera were able to efficiently inhibit the growth of the fungal pathogen *F. oxysporum*. Five isolates from the *B. cereus sensu lato* group displayed a high nematicidal activity on *R. duriopilus* and *P. coffeae*. By displaying a fast growth and a high efficiency against the nematodes, these strains represent good candidates for controlling coffee parasitic nematodes. As perspectives of the current study, further characterizations of the isolates will be performed in order to (i) identify the most efficient isolates up to the species/strain levels, (ii) confirm the absence of human pathogenicity, (iii) characterize the produced compounds/metabolites involved in the nematicidal and antifungal effects and (iv) confirm the promising effects observed *in vitro* with *in planta* experiments.

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CRediT authorship contribution statement

Benoit Duong: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Hoa Xuan Nguyen:** Methodology, Resources, Writing - review & editing. **Ha Viet Phan:** Resources, Writing - review & editing. **Stefano Colella:** Writing - review & editing. **Phap Quang Trinh:** Methodology, Writing - review & editing. **Giang Thi Hoang:** Writing - review & editing. **Tuyet Thi Nguyen:** Methodology, Resources, Writing - review & editing. **Pierre Marraccini:** Writing - review & editing. **Michel Lebrun:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **Robin Duponnois:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126613.

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