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Evaluation of the biocontrol potential of endophytic bacteria isolated from *Coffea liberica* (w. Bull ex hiern) against brown eyespot-causing fungal phytopathogen

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ABSTRACT

Aims: The study aims to investigate the potential of the endophytic bacteria as an alternative to control the devastating brown eyespot disease caused by *Cercospora* sp. in coffee plants. The fungal phytopathogen causes severe leaf fall and berry damages resulting in serious yield losses in coffee farms in the Philippines and worldwide. Currently, the management of this fungal infection relies heavily on synthetic fungicides, which may be of major environmental concern.
Methodology and results: Endophytic bacteria were isolated from the intercellular tissues of *Coffea liberica* leaves by surface sterilization, maceration, dilution technique, plating on trypticase soy agar and colony characterization. Fourteen isolated endophytic bacteria were screened for their ability to inhibit the mycelial growth of *Cercospora* sp. through modified dual culture assay. Isolates HCC10^{-3}SC3, HCC10^{-3}SC2, HCC10^{-1}SC1, ICC10^{-3}SC1, and ICC10^{-1}SC1 yielded the highest percent inhibition of radial growth (PIRG) with 59.56%, 60.92%, 60.96%, 64.36%, and 67.06% respectively and are statistically significant (p<0.05) compared to the antibiotic control nystatin. The top five performing endophytic bacteria were subjected to hydrolytic enzyme production assays and found to exhibit amylolytic, lipolytic, proteolytic, chitinolytic, and cellulolytic activities. Based on the morphological and molecular identification by the 16S rRNA sequence analysis, isolates showed the similarity with *Staphylococcus cohnii*, *Bacillus siamensis*, *Staphylococcus hominis*, and *Kosakonia cowanii* found in GENBANK.
Conclusion, significance and impact of study: The study revealed the biological control potential of endophytic bacteria agents against the brown eyespot-causing fungus in coffee.

Keywords: Endophytic bacteria, *Cercospora* sp., *Coffea liberica*, modified dual-culture assay, 16S rRNA sequencing

INTRODUCTION

Climate change has been reported to favor the occurrence and development of plant phytopathogens in agriculture which in the long run causing dramatic changes in their degree of virulence expressions (Boland *et al.*, 2004), host ranges (Coakley *et al*., 1999), geographical distribution (Shaw and Osborne, 2011), and pathogenicity in a specific region (Elad and Pertot, 2014). Meanwhile, mycotoxins and pesticide residues in food are of major concern associated with a changing climate (Chakraborty and Newton, 2011).

The Philippines is one of the few countries worldwide which can cultivate several commercially important coffee varieties, including the elusive and endangered form of Liberica (*Coffea liberica*) locally known as Kapeng Barako (Department of Trade and Industry, 2017). Because of its lowland-thriving ability, big beans, and robust taste, the demand for this variety in both local and global industry has increased over the years (Davis *et al*., 2019; International Coffee Organization, 2020).

However, the production of *C. liberica* is threatened by various diseases such as the brown eyespot (BES) disease caused by the hyphomycete fungus *Cercospora* spp. (Phengsintham *et al*., 2013). Considered for decades to be of secondary importance (Welles, 1921), BES has increased in severity and re-emerged as a disease of global significance, causing yield loss up to 30% in the nursery to field plantings (Souza *et al*., 2014). The disease is associated with severe leaf fall and reduced berry quality as it induces dark necrotic lesions with a gray-white center and yellow halo on leaves, and black, dry

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elliptical scars on the skin of coffee berries (De Lima *et al*., 2012). The fitting pathogenesis mechanism of *Cercospora* sp. relates to its ability to produce the light-induced mycotoxin, known as cercosporin. Cercosporin generates activated oxygen species, which causes peroxidation of the plant host membrane lipids allowing nutrient leakage for the growth and development of this intercellular pathogen (Daub and Ehrenshaft, 2000). So far, there are no resistant cultivars to BES, and disease management is heavily dependent on the use of synthetic pesticide sprays (Nelson, 2008). However, these pesticides are potentially detrimental to non-target organisms, the environment, and to human health (Damalas and Eleftherohorinos, 2011).

To address the plant diseases caused by fungal pathogens, scientists have put the spotlight on researches for endophytic microorganisms as a potential biological control (Ryan *et al*., 2008; Hardoim *et al*., 2015; Hong and Park, 2016). Endophytes are plant-associated microorganisms that live in plant intercellular tissues such as leaves, roots, seeds, stems, fruits, ovules, and tubers which can directly compete with pathogens as biological control agents (Hallmann *et al*., 1997; Berg *et al*., 2005) and promote hormone-like substances responsible for growth without causing any detrimental effects to their hosts (Ryan *et al*., 2008). Plant inoculation with beneficial endophytes can inhibit disease symptoms caused by viral, insect, fungal, and bacterial pathogens as these microorganisms are known to produce various secondary metabolites and enzymes responsible for their antagonistic ability against phytopathogens (Berg and Hallmann, 2006). Although plant inoculation approach may have varying inhibitory effects against specific phytopathogen, however, the endophyte inoculations can induce abiotic and biotic tolerances of plants amidst climate change (Chakraborty and Newton, 2011).

Exploring the biocontrol potential of endophytic bacteria against *Cercospora* sp. fungal phytopathogen could offer an effective and sustainable way for disease suppression and serve as a natural way to fight the pathogen that attacks the same host. Thus, this study focused on the isolation of endophytic bacteria from *C. liberica* leaves and determined their antifungal activities through modified dual culture assay and hydrolytic enzyme production assay. Most importantly, it aimed to identify the top five performing endophytic bacterial isolates for further testing and evaluate their commercialization value.

MATERIALS AND METHODS

Sample collection

Leaves samples from healthy and infected *C. liberica* were collected from the Department of Agriculture registered tree (NSIC 2007 Cf-L 01'CvSU BARAKO) in the coffee farm of the National Coffee Research Development and Extension Center (NCRDEC), Cavite State University, Indang, Cavite, Philippines (14.1978°N,120.8815°E).

Isolation and morphological characterization of the fungal phytopathogen

The brown eyespot-causing fungal pathogen, *Cercospora* sp., was isolated from the infected leaves of *C. liberica* aseptically. Firstly, the leaves were surface sterilized with 70% ethanol to remove foreign dirt and other contaminants. The infected portion, along with the healthy margin, was cut and inoculated on a potato dextrose agar (PDA), consisting of 20 g dextrose and 15 g agar. The PDA plates were incubated at 25 ± 2 °C and observed for the growth and sporulation of the fungus. The fungus was further purified by agar disc plug method (Surendra *et al*., 2015). A stock culture was made in PDA agar slants and maintained at –20 °C in the culture collection of the Institute for Science and Technology, Center for Life Sciences Research, Microbial Culture Collection, Polytechnic University of the Philippines, Metro Manila, Philippines.

The isolated fungus was macroscopically characterized under a stereomicroscope (Optika, Lombardia, Italy) at 40× magnification. Microscopic characterization was performed by harvesting the conidia from the infected area of the leaf with a sterile needle. The conidia were then mounted on a glass slide with a drop of lactophenol cotton blue and examined under a light microscope (with 1000× magnification) (Mohammadpourlima *et al*., 2017). Morphological species characterization was confirmed following the key characters as described by previous studies (Liberato and Shivas, 2006; Phengsintham *et al*., 2013).

Isolation and morphological characterization of endophytic bacteria

The leaves of *C. liberica* were surface sterilized with 70% ethanol and 2% sodium hypochlorite and soaked three times in sterile distilled water. The isolation of endophytic bacteria was performed in triplicates. The leaves were cut into small pieces and were macerated using a sterile mortar and pestle. A total of 1 g macerated leaves was transferred into a centrifuge tube with 9 mL of natural saline solution (NSS). After homogenizing, the mixture was plated onto a trypticase soy agar (TSA) for each dilution (10^{-1}, 10^{-2} and 10^{-3}) and incubated at 37 °C for 24 h. The bacterial colonies on the mixed plate were selected according to their morphology (size, color, shape) and sub-cultured by quadrant streak method. A stock culture was made in tryptic soy broth (TSB) consisting of 17 g tryptone, 3 g soytone, 2.5 g glucose, 5 g sodium chloride (NaCl), 2.5 g dipotassium phosphate and incubated at 37 °C for 24 h. A 15% glycerol was added, and the cultures were maintained in the freezer at –20 °C (Costa *et al*., 2012; Silva *et al*., 2015).

Modified dual culture assay of endophytic bacteria against fungal phytopathogen

The antifungal activity of the endophytic bacteria against *Cercospora* sp. fungal phytopathogen was investigated

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following the described method by Muleta *et al*. (2007). Each bacterial isolate was sub-cultured into TSB aseptically and incubated at 37 °C for 24 h. The optical density of the bacterial broth suspension was adjusted to approximately 1.5×10^8 CFU/mL to match 0.5 McFarland standard (Clinical and Laboratory Standards Institute, 2018). The fungal phytopathogen, on the other hand, was prepared by sub-culturing the isolated *Cercospora* sp. onto sterile PDA plates and incubated at 25 ± 2 °C for 72 h. Then, a 5 mm agar disc from the leading margin of the fungal culture plate was obtained using a sterile cork borer and placed onto the TSA-PDA plate. Afterward, each of the previously standardized endophytic bacterial cultures was streaked as broadband (making a straight short bar) using sterile applicator sticks 20 mm from the opposite edge of the agar plate. The plate inoculated with the test fungus alone served as the positive-growth control. Nystatin, an antifungal drug was used as antibiotic control. The plates were incubated at 25 ± 2 °C and observed for 216 h and potent endophytic bacterial isolates were selected by measuring the fungal radial growth using a Vernier scale and by computing the percent inhibition of radial growth (PIRG) (Rahman *et al*., 2009).

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

*R*1 = Radial growth of fungal colony with no treatment (mm)
*R*2 = Radial growth of fungal colony with endophytic bacterial treatments (mm)

Hydrolytic enzyme assay of endophytic bacteria

To determine the ability of the endophytic bacterial isolates to produce hydrolytic enzymes which may be attributed to their antifungal properties, hydrolytic enzyme plate assays were conducted in triplicates. The presence of enzymes was indicated by the zones of clearance around the colonies of the bacteria. Visualization of hydrolysis around the colony was aided by adding appropriate stains and the zones of clearance was measured with a Vernier scale (Muñoz *et al*., 2014; Latorre *et al*., 2016).

Detection of amylolytic activity

Amylolytic activity test was conducted to determine the ability of endophytic bacteria to hydrolyze the starch. Briefly, starch agar was prepared by mixing 0.6 g beef extract, 2.0 g soluble starch, and 2.4 g agar in 200 mL distilled water. Then, the bacterial isolate was stabbed in the agar plates and incubated at 37 °C for 48 h. The staining reagent, 1% iodine solution was then poured onto the plates, and distributed using a platform shaker for 5 mins. The zone of clearance formed around the colony after staining indicating hydrolysis was measured with a Vernier scale (Latorre *et al*., 2016).

Detection of lipolytic activity

Lipolytic activity test with modification was performed to determine the ability of endophytic bacteria to hydrolyze lipids and fats. The Tween 20 agar was made by mixing 10 g peptone, 5.0 g NaCl, 0.1 g calcium chloride hydrate ($CaCl_2 \cdot H_2O$), 20 g agar and 10 mL Tween 20 diluted in 1000 mL distilled water. The bacterial isolates were stabbed in the agar plates and incubated at 37 °C for 48 h. To facilitate visualization of calcium salt precipitate indicative of hydrolysis reaction, methylene blue was poured onto the plates and distributed using a platform shaker for 5 min. After staining, the zone of clearance that formed around the colony indicating hydrolsis was measured with a Vernier scale (Latorre *et al*., 2016).

Detection of proteolytic activity

Proteolytic activity test was conducted to investigate the ability of endophytic bacteria to break down proteins and hydrolyze peptides. Modified skim milk agar was prepared by mixing 2.8 g skim milk powder, 0.5 g casein enzyme hydrolysate, 0.25 g yeast extract, 0.1 g dextrose and 1.5 g agar in 100 mL distilled water. The bacterial isolates were then stabbed in the agar plates and incubated at 37 °C for 48 h. The zone of clearance formed around the colony indicative of proteolytic activity was measured with a Vernier scale (Latorre *et al*., 2016).

Detection of cellulolytic activity

The cellulolytic activity test was performed to determine the ability of endophytic bacteria to hydrolyze cellulose. The modified carboxymethyl cellulose (CMC) agar medium was made by mixing 0.2 g ammonium dihydrogen phosphate ($NH_4H_2PO_4$), 0.04 g potassium chloride (KCl), 0.25 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H2O$), 0.6 g TSA, 3.0 g carboxymethyl cellulose and 3.0 g agar diluted in 200 mL distilled water. The bacterial isolates were stabbed in the agar plates and incubated at 37 °C for 48 h. The staining reagent, Congo red, was poured onto the agar, distributed using a platform shaker for 5 min and washed off with NSS to view the zone of clearance indicative of cellulase activity and was measured with a Vernier scale (Muñoz *et al*., 2014).

Detection of chitinolytic activity

Chitinolytic activity test with modification was performed to determine the potential of endophytic bacteria to break down chitin and fungal cell walls. The colloidal chitin agar was prepared by mixing 2.5 g colloidal chitin, 0.15 g peptone, 0.15 g yeast extract, 0.35 g dipotassium phosphate (K_2HPO_4), 0.15 g monopotassium phosphate (KH_2PO_4), 0.25 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 1.0 g amino nitrate (NH_2NO_3), 7.5 g agar, and 0.5 g NaCl diluted in 500 mL distilled water. The bacterial isolates were stabbed in the agar plate and incubated at 37 °C for 48 h. The staining reagent, crystal

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violet, was poured onto the agar plates, distributed using a platform shaker for 15 minutes and washed off with NSS to view the zone of clearance, indicative of chitinolytic activity (Zarei *et al*., 2011).

Statistical analysis

All data were recorded, edited, and processed by the One-Way Analysis of Variance (ANOVA) at 0.05 level of significance to determine if there is a significant difference between the means followed by posthoc test (independent t-test) to determine where the significance lies within the means of the data using SPSS Statistics 20 (IBM Corp., New York, USA).

Molecular identification of endophytic bacteria by 16s rRNA sequence analysis

The top five performing endophytic bacterial isolates were cultivated onto TSA media for 24 h at 37 °C. A single colony of bacteria was acquired using a sterile cork borer and transferred to a microcentrifuge. The samples were sent to MACROGEN, Korea for DNA extraction and 16S rRNA sequencing. Basic Local Alignment Search Tool (BLAST) was used to compare the similarity of nucleotide sequences from the standard report to the sequences in the database (Madden, 2013). The sequences were extracted from BLAST and were processed in SeaView software (Gouy *et al*., 2009). The phylogenetic tree was constructed using Molecular Evolutionary Genetics (MEGA) Analysis 6.0 bootstrapped 1000 times (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Cercospora species, belonging to the genera of hyphomycetes, is one of the most important phytopathogenic fungi in the agricultural field that causes necrotic spots and lesions on leaves, fruits, flowers, and seeds on numerous hosts in most climatic regions. The success of these groups of pathogens is not only attributed to their airborne mode of transmission and widespread distribution but also the susceptibility of several agricultural commercial crops to this pathogen (To-Anun *et al.*, 2011). Among the most destructive are *C. beticola* on sugar beet causing leaf spot (Vereijssen *et al*., 2007); *C. kikuchii* on soybean causing purple seed stain (Imazaki *et al*., 2007); *C. zeae-maydis* on corn causing grey leaf spot (Crous *et al*., 2006) and *C. coffeicola* on coffee causing brown eyespot (Souza *et al*., 2010). Brown eyespot disease caused by *Cercospora* sp. is one of the major diseases of coffee-growing regions of the world including Ethiopia (Van der Graaff and Pieters, 1983), Brazil (Martins *et al*., 2008), Mexico (Ayala-Escobar *et al*., 2009), Hawaii (Nelson, 2008), India (Meghvansi *et al*., 2013), and the Philippines (Welles, 1921).

Morphological characterization of the fungal phytopathogen

Cercospora species exhibit host-specificity which means one species, genus, or family of the host plant is equal to one *Cercospora* species (Chupp, 1954). For years, morphological characterization and the relationship of cercosporoid fungi with their host plants are widely used in establishing a taxonomic description (Groenewald *et al*., 2013). Therefore, the study used the existing *Cercospora* sp. macroscopic, microscopic, cultural characteristics and host specificity to confirm the identity of the fungus isolated from *C. liberica* leaves as *Cercospora* sp.

The macroscopic morphology viewed under a stereomicroscope at 40× magnification showed spots that are yellow to light brown (Figure 1A). As time progressed, the spots became small to fairly, large, and suborbicular to irregular in shape (Figure 1B). The center portion turned grey and dark (Welles, 1921; Souza *et al.*, 2010) and exhibited concentric striations encircled by a yellowish halo (Phengsintham *et al.*, 2013). Figure 1C shows the cultural characteristics of the fungus on PDA after 216 h of incubation at 25 ± 2 °C showing colonies that are white in the center, cottony, flat, irregular in shape and had a green to dark brown pigmentation in the margin (Phengsintham *et al.*, 2013).

Besides, the fungal structures of *Cercospora* sp. acquired from the leading margin of the colony in plates, stained with lactophenol cotton blue and viewed under 1000× magnification with the light microscope (Figure 1D) also showed similarity with those described in previous studies (Liberato and Shivas, 2006; Phengsintham *et al.*, 2013) on the microscopic morphological characteristics of *Cercospora* species. Conidia are septate, hyaline, solitary, acicular to obclavate, straight to curved, thin-walled smooth, tip acute, and base truncate to obconical truncate.

Isolation and morphological characterization of endophytic bacteria

Bacterial endophytes are naturally occurring beneficial microorganisms in plant intercellular tissues that creates a symbiotic relationship as they can act as biological control agents of phytopathogens which promote plant growth and nutrition (Hong and Park, 2016). Endophytic bacteria are present inside various plant tissues such as leaves, roots, seeds, stems, fruits, ovules, and tubers (Hallman *et al*., 1997), but the highest endophytic bacterial population density is present mainly in the rhizosphere (roots). However, in this study, endophytic bacteria were only isolated from the phyllosphere or leaf intercellular tissues of *C. liberica* to target the *Cercospora* sp. which is an intercellular leaf inhabiting microorganism by nature (Souza *et al*., 2010).

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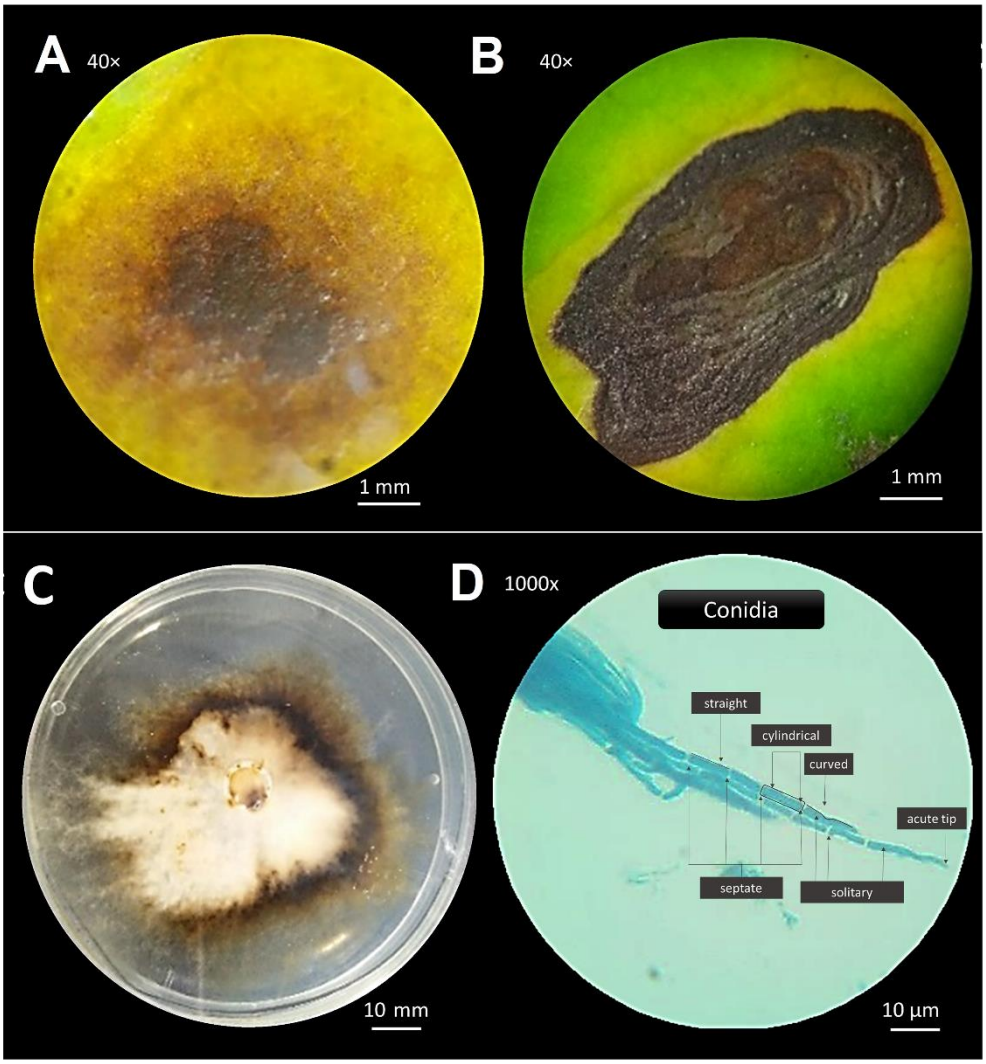

Figure 1: Macroscopic, cultural, and microscopic characteristics of the *Cercospora* sp. fungal pathogen. (A-B) Brown eye spot on *C. liberica* leaf; (C) fungal culture on PDA agar; and (D) conidia stained by lactophenol cotton blue viewed under 1000× magnification.

A total of 14 endophytic bacteria were isolated from the leaf samples. The present findings on the isolation of bacteria from the coffee leaves confirm the presence of bacterial species in the intercellular spaces of coffee plant tissues, as demonstrated also in previous studies (Wilson, 1995; Saikkonen *et al*., 2004; Vega *et al*., 2005). No growth was seen in the sterility check experimental setups confirming the efficiency of the surface disinfestation procedure and the complete recovery of the internal population of bacteria from the intercellular leaf tissues of *C. liberica.* However, the concentration of endophytic bacteria in CFU/mL considering the dilution factor was not known because only 14 endophytic bacterial colonies were developed on the TSA agar. The result indicates that there might be a low population density of endophytic bacteria in the phyllosphere (leaf tissues) of *C. liberica* or the trituration and surface disinfestation protocol performed was successful resulting in the reduction in total numbers of the indigenous endophytic community (Hallman *et al*., 1997).

Table 1 shows the results of the Gram and endospore staining. Total of 12 isolated bacterial species are Gram-positive; four of which produced endospores. While two out of the 14 endophytic isolates are Gram-negative bacteria. Figure 2 shows the macroscopic and microscopic characteristics of one of the isolated Gram-positive endophytic bacterium, HCC10^{-3}SC3. Confluent growth on solid medium (Figure 2A), with. irregular, dry, matte and brittle, with white to yellow color colonies (Figure 2B) were observed after 24 h of incubation. Gram-positive bacteria indicate the presence of a thick layer of peptidoglycan in the cell wall while an endospore positive bacterial imply the bacteria's potential ability to adapt and survive in an adverse condition (Atrih *et al*., 1999).

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Table 1: Summary of the microscopic characterization of the endophytic bacteria isolated from the leaves of *C. liberica*.

Isolates	Gram Staining	Endospore Staining	Shape
HCC10^{-1}SC1	+	-	Spherical in clusters
HCC10^{-1}SC2	+	-	Spherical
HCC10^{-1}SC3	+	-	Spherical
HCC10^{-1}SC4	+	+	Rod-shaped
HCC10^{-3}SC1	+	+	Rod-shaped
HCC10^{-3}SC2	+	+	Rod-shaped
HCC10^{-3}SC3	+	+	Rod-shaped
ICC10^{-1}SC1	+	-	Spherical in clusters
ICC10^{-1}SC2	+	-	Short rod-shaped
ICC10^{-1}SC3	+	-	Spherical
ICC10^{-1}SC4	+	-	Short rods
ICC10^{-2}SC1	-	-	Rod-shaped
ICC10^{-2}SC2	+	-	Spherical in chains
ICC10^{-3}SC1	-	-	Rod-shaped

(+) positive reaction; (-) negative reaction.

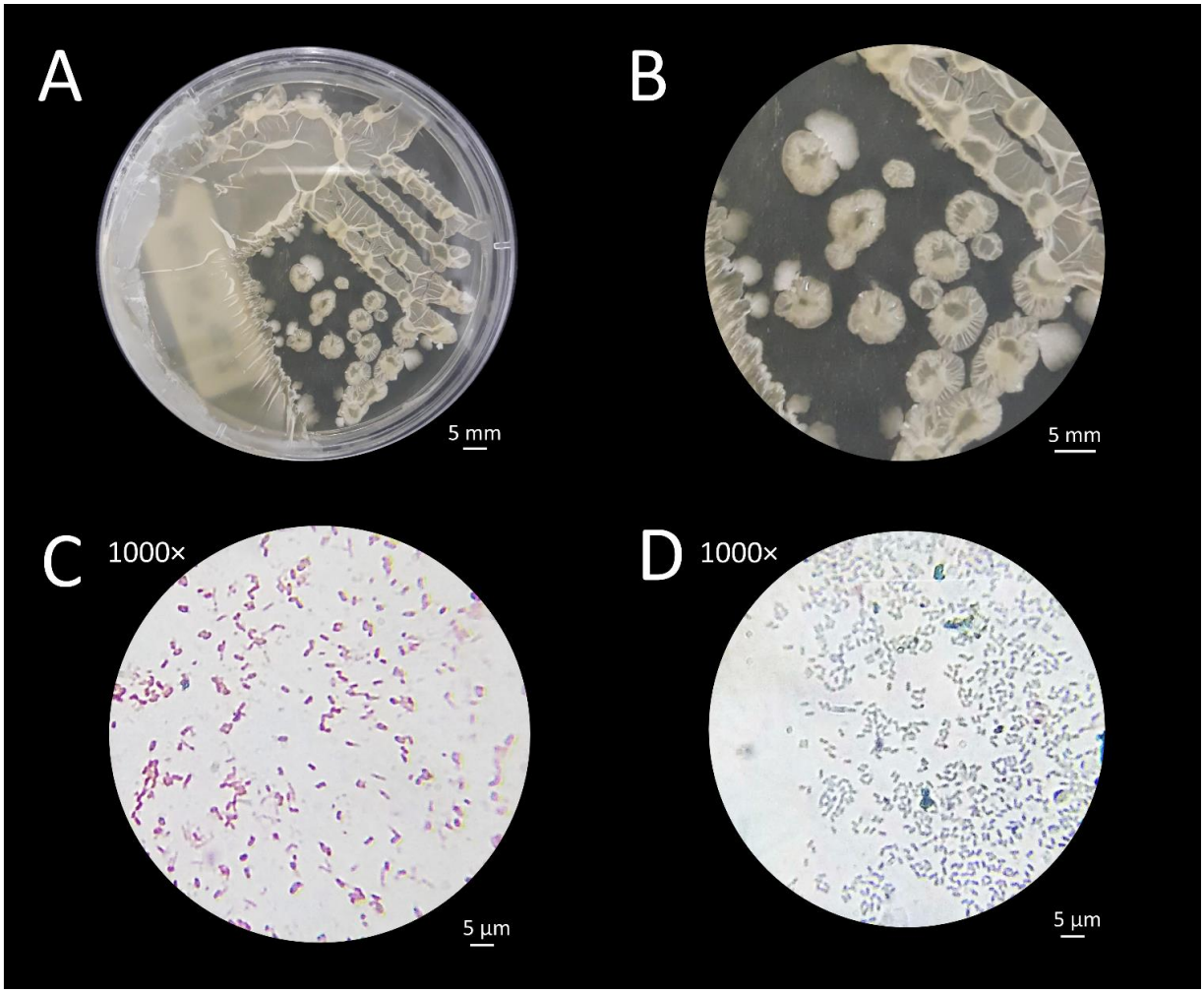

Figure 2: Macroscopic and microscopic characteristics of the endophytic isolate HCC10^{-3}SC3. (A-B) Colonies on TSA agar after 24 h of incubation at 37 °C. (C) Gram stain showing purple rod-shaped bacterial cells viewed under 1000× magnification. (D) Endospore stain showing green, spherical bodies stained with malachite green viewed under 1000× magnification.

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Modified dual culture assay of endophytic bacteria against fungal phytopathogen

Modified dual culture assay was conducted to know the ability of the 14 endophytic bacteria to inhibit the mycelial growth of *Cercospora* sp. on TSA-PDA agar incubated at 25 ± 2 °C for 216 h. Compared to the control group, treatment with endophytic bacteria yielded a smaller mycelial radial growth (Figure 3). Reduced pigmentation on the mycelia of *Cercospora* sp. with endophytic treatment was also observed indicating that the cercosporin, the polyketide toxin responsible for its mode of pathogenicity, was also reduced (Mitchell *et al*., 2002; You *et al*., 2008). The 14 endophytic bacterial isolates tested for *in vitro* antifungal activity against *Cercospora* sp. were found to inhibit the mycelial growth of *Cercospora* sp. with varying efficacies. Thirteen of the isolates showed statistically significant inhibitory activity as compared to antibiotic control (p<0.05) (Table 2). The percentage inhibition of radial growth by the endophytic bacterial isolates against *Cercospora* sp. varied between 41.53% and 67.06% (Table 2), which suggests that the endophytic bacterial isolates produce various antagonistic chemical substances against *Cercospora* sp. (Berg *et al*., 2005; Rahman *et al*., 2009). The endophytic bacterial isolates that caused significant radial mycelial growth inhibition were not in physical contact and did not overlap with the pathogen, thus, indicating that the isolates released certain antifungal metabolites into the agar medium (Montealegre *et al*., 2003). Modified dual culture assay revealed the top five performing endophytic bacterial isolates with the lowest average radial mycelial growth and highest percent inhibition of radial growth (PIRG) as ICC10^{-1}SC1 (67.06%), ICC10^{-3}SC1 (64.36%), HCC10^{-1}SC1 (60.96%), HCC10^{-3}SC2 (60.92%), and HCC10^{-3}SC3 (59.56%).

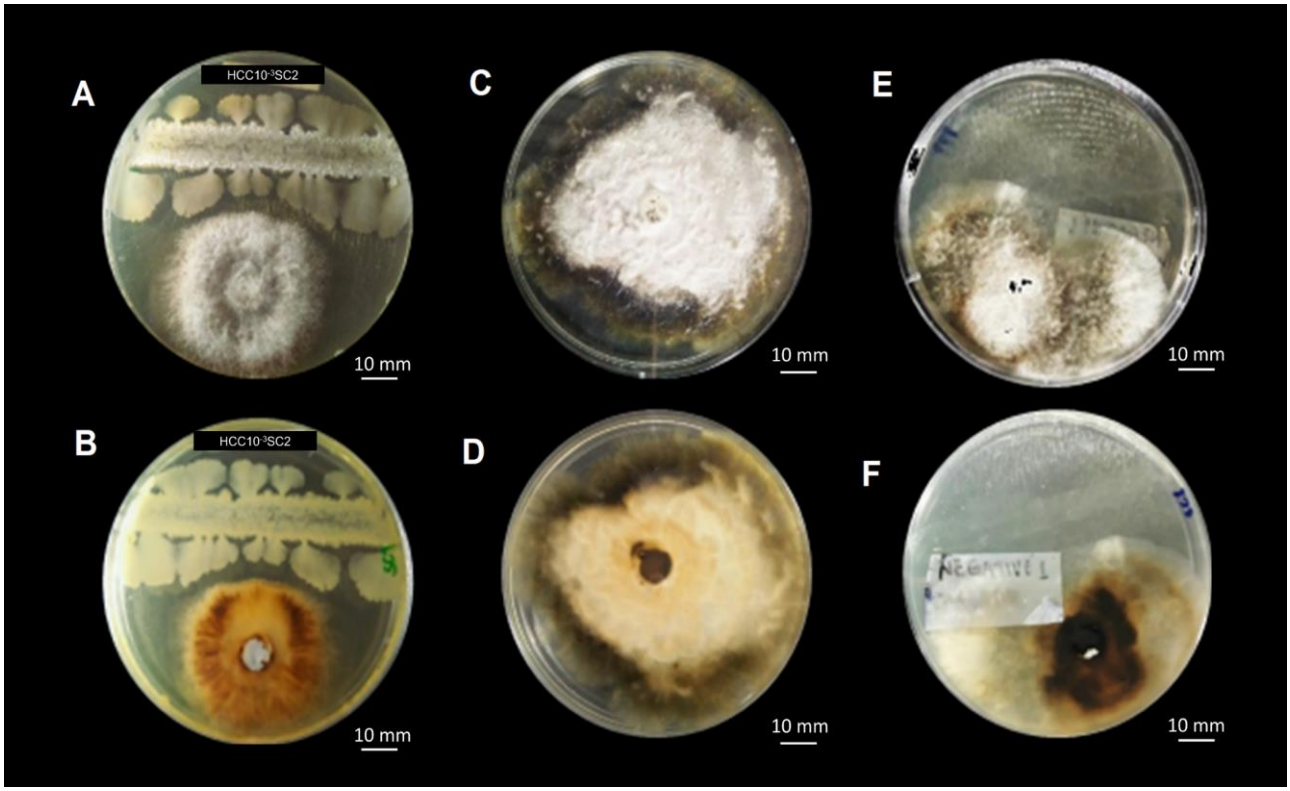

Figure 3: Modified dual culture on PDA-TSA agar after 216 h of incubation at 25 ± 2 °C. (A-B) Endophytic bacterial treatment using isolate HCC10^{-3}SC2; (C-D) positive-growth control (without endophytic bacterial treatment), and (E-F) antibiotic control.

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Table 2: Average mycelial radial growth and percent inhibition of radial growth (PIRG) of the 14 endophytic bacteria.

Isolate	Average radial growth (mm)	PIRG (%)	Isolate	Average radial growth (mm)	PIRG (%)
HCC10^{-1}SC1	19.11 ± 0.92	60.96	ICC10^{-1}SC1	16.12 ± 1.35	67.06
HCC10^{-1}SC2	20.99 ± 0.36	57.12	ICC10^{-1}SC2	21.87 ± 2.67	55.34
HCC10^{-1}SC3	25.26 ± 0.51	48.42	ICC10^{-1}SC3	25.88 ± 1.11	47.14
HCC10^{-1}SC4	21.03 ± 1.25	57.03	ICC10^{-1}SC4	25.81 ± 2.02	47.27
HCC10^{-3}SC1	22.57 ± 0.65	53.89	ICC10^{-2}SC1	28.63 ± 0.59*	41.53
HCC10^{-3}SC2	19.13 ± 0.22	60.92	ICC10^{-2}SC2	26.28 ± 2.14	46.32
HCC10^{-3}SC3	19.80 ± 0.31	59.56	ICC10^{-3}SC1	17.45 ± 0.87	64.36
Positive-growth control (without endophytic bacterial treatment)	48.95 ± 1.93	-	Antibiotic control (Nystatin)	45.59 ± 1.43	6.86

*Average radial growth of the isolate not statistically significant (p<0.05) as compared to the antibiotic control (nystatin).

Hydrolytic enzyme assay of endophytic bacteria

The top five performing endophytic bacteria were subjected to hydrolytic enzyme production assay to screen their ability to produce hydrolytic enzymes that can trigger hydrolysis of the specific bodily compounds of the pathogen. The ability to produce these enzymes is associated with the endophytic bacteria's mode of antagonism, resulting in fungal nutrient deprivation and reduced growth (Junaid *et al*., 2013). The endophytic bacterial isolates exhibited clear and gradient zones around colonies (Figure 4), which implies their ability to produce hydrolytic enzymes (Latorre *et al*., 2016). Table 3 shows the hydrolytic enzyme production of the top five performing endophytic bacteria. ICC10^{-1}SC1, ICC10^{-3}SC1, HCC10^{-1}SC1, HCC10-^{3}SC2, and HCC10-^{3}SC3 reveal positive activities in the amylolytic, lipolytic, proteolytic and chitinolytic enzymes assay forming small to large halos around colonies which indicates that they can hydrolyze the starch and carbohydrates; degrade lipid and fats; break down proteins (Latorre *et al*., 2016) and degrade the chitin (Zarei *et al*., 2011) of the fungal pathogen respectively. Positive activities of HCC10^{-1}SC1 and ICC10^{-3}SC1 in the cellulolytic enzyme assay confirmed the ability of these bacteria to hydrolyze the cellulose and other polysaccharides (Muñoz *et al*., 2014) resulting in fungal antagonism. The positive activities in the enzymatic assays confirmed the antifungal properties of the top five performing endophytic bacterial isolates (Silva *et al*., 2015).

Molecular identification of endophytic bacteria by 16s rRNA sequence analysis

The NCBI Blast hits results are shown in Table 4. The isolate HCC10^{-1}SC1 exhibited 99% sequence similarity to *Staphylococcus cohnii*, HCC1010^{-3}SC2 and HCC10^{-3}SC3 exhibited 99% sequence similarity to *Bacillus siamensis,* ICC10^{-1}SC1 exhibited 99% sequence similarity to *Staphylococcus hominis* and ICC10^{-3}SC1 exhibited 99% sequence similarity to *Kosakonia cowanii.* The phylogenetic tree was constructed according to the 16S rRNA sequence analyses show the relationship between the endophytic bacterial isolates and their representative species where *Candida albicans* serve as the outgroup control (Figure 5). The neighbor-joining tree shows that isolates HCC10^{-1}SC1 and ICC10^{-1}SC1 belong to the *Staphylococcus* spp.; HCC10^{-3}SC2 and HCC10^{-3}SC3 belong to *Bacillus* spp. and ICC10^{-3}SC1 belongs to *Enterobacter* spp. The gene sequences were registered in the Genbank of NCBI acquiring accession gene IDs MK574683 and MK574684 for *Bacillus siamensis*, MK574682 for *Staphylococcus cohnii*, KY451837 for *Kosakonia cowanii* and MK574685 for *Staphylococcus hominis.*

Table 3: Hydrolytic enzyme production of the top five performing endophytic bacteria.

Isolates	Amylase	Lipase	Protease	Chitinase	Cellulase
HCC10^{-1}SC1	++	++	+++	+	+
HCC10^{-3}SC2	+++	++	+++	++	-
HCC10^{-3}SC3	+++	+++	+++	+++	-
ICC10-^{1}SC1	+++	+++	+++	++	-
ICC10^{-3}SC1	++	+++	+++	+++	+

(+) zone of clearance of <5 mm; (++) zone of clearance of 5-10 mm; (+++) zone of clearance of >10 mm; (-) absence of zone of clearance.

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Malays. J. Microbiol. Vol 16(6) 2020, pp. 477-490
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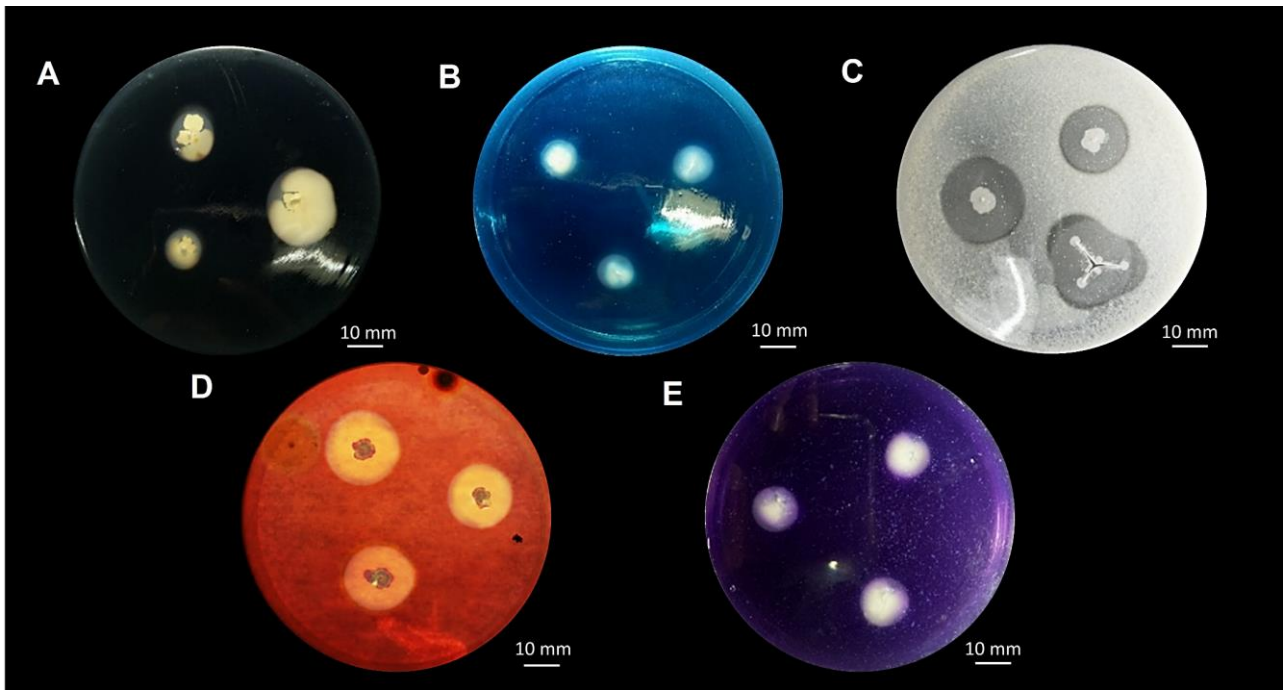

Figure 4: Hydrolytic enzyme production of endophytic bacteria isolated from *C. liberica* leaves. (A) Amylase positive endophytic bacterial isolate HCC10^{-1}SC1 on starch agar; (B) lipase positive endophytic bacterial isolate HCC10^{-3}SC2 on Tween 20 agar; (C) protease positive endophytic bacterial isolate HCC10^{-3}SC3 on skimmed-milk agar; (D) cellulase positive endophytic bacterial isolate ICC10^{-3}SC1 on carboxymethyl cellulose agar, and (E) chitinase positive endophytic bacterial isolate ICC10-^{1}SC1 on colloidal chitin agar.

Table 4: NCBI BLAST hits results of the consensus sequences of the top five endophytic bacteria.

Code	Organism	Query Coverage (%)	E-value	Identity (%)	NCBI Accession
HCC10^{-1}SC1	*Staphylococcus cohnii*	98	0	99	MK574682
HCC10^{-3}SC2	*Bacillus siamensis*	98	0	99	MK574683
HCC10^{-3}SC3	*Bacillus siamensis*	99	0	99	MK574684
ICC10-1SC1	*Staphylococcus hominis*	99	0	99	MK574685
ICC10^{-3}SC1	*Kosakonia cowanii*	95	0	99	KY451837

Species under the genus *Staphylococcus* are found to exhibit biological control properties as they can induce antagonism against pathogens while promoting growth to the host plant (Labuschagne *et al*., 2010). In this study, *S. hominis* and *S. cohnii,* in particular*,* have high percent inhibition of radial growth against *Cercospora* sp. and are positive to several hydrolytic enzyme productions which may cause major fungal structure degradation and reduced nutrition of fungal pathogen (Wargo and Hogan, 2006). *Staphylococcus hominis* was first to be isolated inside the seed tissues of rice and confirmed to be an endophytic bacterium in nature (Chaudhry *et al*., 2017). The endophytic bacteria was proven to provide benefits to its host plants as it contains genes promoting protection against stress and could produce siderophores supplying the plant with iron and other nutrients, and auxin which is a plant growth-promoting hormone (Chaudhry *et al*., 2017). *Staphylococcus cohnii* subsp. *cohnii* (Kloos and Wolfshohl, 1991) is a Gram-positive bacterium that was also found to be endophytic in rice seeds variety along with *S. hominis* (Chaudhry *et al*., 2017). This endophytic bacterium has revealed to provide positive effects to its host plants such as stress tolerance supplying the plants with substances that alleviate oxidative and heat stress. It also releases auxin and siderophores which are mainly responsible for the growth, nutrition, and protection of the plants (Chaudhry *et al*., 2017). Although these *Staphylococcus* species show promising results as potential biocontrol agents against *Cercospora* sp., they are considered opportunistic which may cause minimal to fatal infections with patients having weak immune systems (Jiang *et al*., 2012). Thus, the introduction of these bacteria as live microorganisms in the environment to control the phytopathogen might be a problem due to the hazard that they might bring to humans.

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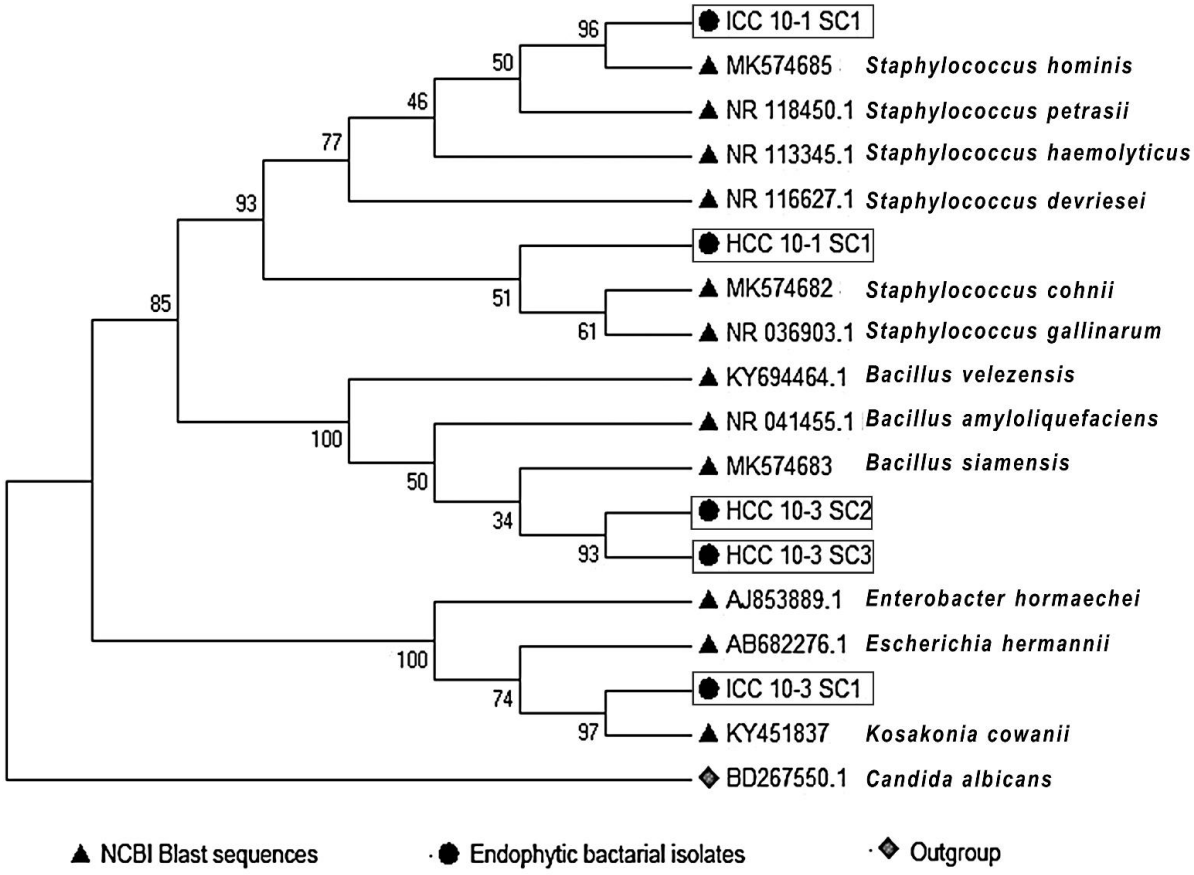

Figure 5: Neighbor-joining tree of the top five performing endophytic bacterial strains based on the 16S rRNA gene sequences comparison. *Candida albicans,* a fungus, was used as an outgroup.

Some species belonging to the genus *Enterobacter* are also found to impose beneficial effects on plants (Deepa *et al*., 2010; Ramesh *et al*., 2014) and induce resistance mechanisms to plant tissues. In this study, *Kosakonia cowanii* (formerly known as *Enterobacter cowanii*) was found to have a wide range of hydrolytic enzymes and high antagonistic activity against *Cercospora* species. According to studies, members of the genus *Kosakonia* also promote plant growth by releasing stress-reducing substances and forming siderophores that supply beneficial iron and other nutrients helping the plant tissues to perform bodily functions (Rana *et al*., 2011; Menendez and Garcia-Fraile *et al*., 2017). They are Gram-positive bacteria that were found to be endophytic in plants. *Kosakonia cowanii,* together with other *Enterobacter* species, has been observed for its remarkable plant growth promoting activity by producing nitrogen-fixing substances (Lin *et al*., 2012). Its closely related strain, *K. oryzae,* is also revealed to induce auxin and produce siderophores which are primarily responsible for the plants' nutrition and immunity against diseases (de Souza *et al*., 2012). However, like the *Staphylococcus* spp., this isolate is also a human pathogen first to be isolated in the blood of a human patient (Yang *et al*., 2018).

Bacillus spp*.* are widely used as a biocontrol in plant diseases (Fravel, 2005). These species have been investigated for the production of a wide range of biocontrol compounds and secondary metabolites used to combat pathogens especially peptides and lipopeptides; catalysts in biosynthetic mechanisms (Fira *et al*., 2018). In this study, two isolated *Bacillus siamensis* showed promising results with 59.56% and 60.92% percent inhibition of radial growth and positive activities in the amylolytic, lipolytic, proteolytic and chitinolytic enzymatic assay which confirmed their potential as biocontrol agents against *Cercospora* sp. According to the study of Meidong *et al*. (2017), *Bacillus siamensis* is a novel Gram-positive endophytic genus of bacteria first isolated from processed salted crab products in Thailand. This endophytic bacterium initially showed potential as a probiotic in

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aquaculture showing antagonistic ability against fish pathogens. In a different study, it was found to inhibit the growth of the mycelia of fungal pathogen *Rhizoctonia solani* from plants (Jeong *et al*., 2012). Furthermore, it was revealed that *B. siamensis* can produce bacteriocin-like activity that inhibits the growth of the pathogen through its active proteins (Meidong *et al*., 2017). *Bacillus* spp. can produce biofilms and several hydrolytic enzymes such as protease and cellulase which are key enzymes to degrade proteins, peptides, and other polysaccharides (Sumpavapol *et al*., 2010). On the other hand, *B. siamensis* could also provide benefits to plants as it promotes plant growth through volatile organic compounds emission (Tahir *et al*., 2017). Considering the high antagonism of *B. siamensis* against *Cercospora* sp. and the presence of various hydrolytic enzymes based on the results yielded; the profile of the bacteria as being endophytic and probiotic by nature and its relatively less toxicity to humans and the environment. Therefore, *B. siamensis* is the most promising isolate for the biocontrol of *Cercospora* sp. ravaging coffee plants.

CONCLUSION

A total of 14 endophytic bacteria were isolated from *C. liberica* leaves. The modified dual culture assay revealed that the isolates have high antagonistic efficacy of 41.53% to 67.06% against the brown eyespot causing *Cercospora* sp. fungal pathogen, with some that are superior to the antibiotic solution nystatin. Morphological and molecular identification revealed the identities of the top five most promising endophytic bacteria as *Staphylococcus cohnii,* two *Bacillus siamensis*, *Staphylococcus hominis* and *Kosakonia cowanii*.

The antifungal properties of these five endophytic bacteria may be due to the presence of hydrolytic enzymes such as amylase, lipase, protease, cellulase and chitinase with specific mode of actions which are not detrimental to the host plant but may affect the growth and life cycle of the fungal pathogen instead. The inhibitory effect and ability to produce a wide range of hydrolytic enzymes suggest that these endophytic bacteria can be used as a microbial inoculant for the control of brown eyespot disease caused by *Cercospora* sp. fungal pathogen in *C. liberica* coffee plants. More tests shall be conducted to unravel the bioactivities of the endophytic bacteria in the greenhouse and field conditions; and their antibacterial and antifungal effects against other coffee phytopathogens.

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