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Bacteria communities of coffee plant rhizosphere and their potency as plant growth promoting

SUHARJONO[1,♥], ERVINDA YULIATIN[2]

[1]Department of Biology, Faculty of Science, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia. Tel./fax.: +62-341-575841, [♥]email: csharjono@gmail.com

[2]Department of Biology, Faculty of Mathematics and Natural Science, Universitas Mulawarman. Jl. Barong Tongkok No. 4, Gunung Kelua, Samarinda 75119, East Kalimantan, Indonesia

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Abstract. *Suharjono, Yuliatin E. 2022. Bacteria communities of coffee plant rhizosphere and their potency as plant growth promoting. Biodiversitas 23: 5822-5834.* This study aimed to investigate the soil bacteria communities of the coffee rhizosphere and evaluate the potency in supporting coffee plant growth. The soil was collected from the *Coffea canephora* and *Coffea arabica* in Malang, East Java. The bacterial genomic DNA was extracted by FastDNA Spin kit while the Illumina platform analyzed the total gDNA. Other samples were isolated using the serial dilution method on Tryptic Soy Agar, Pikovskaya Agar, and Nitrogen-free-Bromothymol Blue Agar medium to isolate IAA-producing, phosphate-solubilizing, and nitrogen-fixing bacteria, respectively. The selected bacteria isolates were identified based on 16S rDNA sequencing. As a result, the Proteobacteria showed dominance at the phyla level, and *Bradyrhizobium elkanii* was the most abundant species with a not significant different proportion between Robusta and Arabica soil. The quantifying method of the selected isolate showed the maximum concentration, such as S1.6.3.2 isolate producing IAA-hormone at 104.46 µg/mL, W3.5 isolate solubilizing the phosphate at 4.5 µg/mL, and W1.2 isolate fixing the ammonia at 21.54 µg/mL. Those potential isolates, S1.6.3.2, W3.5, and W1.2, were identified as *Bacillus subtilis* DSM 10, *Pseudomonas putida* S18, and *Bacillus methylotropicus* SY2, respectively. Further research shows that those bacteria consortiums can be a candidate as biofertilizers due to helping soil health stimulation and promoting coffee growth.

Keywords: *Bradyrhizobium*, coffee, genomic, rhizosphere, soil

INTRODUCTION

Population growth in the world will make the crop production demand double by 2050. As a result, coffee consumption has risen almost 200 times in nearly six decades (Caldwell et al. 2015). Moreover, coffee is an essential commodity in the international market, a substance source for more than 125 million people in Asia, Africa, and Latin America (Organization 2014). In Indonesia, coffee is a vital commodity, either local or global market, that contributes to Indonesia's income. Generally, *Coffea arabica* and *Coffea canephora* are widely planted coffee species, produced by global production at 70% and 30%, respectively. Both variants are favorable tastes for coffee addicts, which affected the coffee bean production increase to serve coffee consumer demand (World Coffee Research 2017).

Nowadays, coffee beans with various tastes are increasing faster, especially coffee production in Malang, East Java. Universitas Brawijaya Forest (UBF), Malang, have been developed an eco-friendly forest-based coffee plantation. However, coffee productivity in 2017-2018 decreased due to high rain sessions and unstable soil fertility in UBF. The UBF practicians and local farmers have utilized manure fertilizer (compost) to promote its growth and production but resulted in no significant changes.

In combating this problem, we conducted a preliminary study on soil physicochemical properties in the coffee plantation area. The result showed that the soil was very acidic (pH at 4.0-4.5), and the bacteria colony was 10^4-10^5 CFU/g (Yuliatin et al. 2019). According to Good Agriculture Practices (GAP) for coffee growth, the suitable pH is 5.5 to 6.5 for the coffee plantation area (KEMENTAN 2014), and the bacterial colony concentration indicated that the soil was under stress environment (Urgiles-Gómez et al. 2021). Therefore, we predicted those areas could be improved using a specific method, such as exploring the indigenous bacteria around the coffee rhizosphere. Various techniques for improving coffee production without destroying soil health, such as formulating the biofertilizer as eco-friendly fertilizer mediated by consortium microbes and enhancing understanding of genetic control of plant-microbe interaction (Cenci et al. 2012) and bacteria community in the soil (Beule and Karlovsky 2021). The coffee growth can be improved by bacteria associated with rhizosphere coffee using biofertilization by increasing nutrient availability such as N or P (Urgiles-Gómez et al. 2021). Bacteria diversity is vital as a bioindicator of soil health; the more diverse the microbe, the more healthy the soil. However, the presence of soil microbes, such as bacteria surrounding the plant root, will cause an imbalance (Zhong et al. 2020); hence those trigger pathogen bacteria in the soil to spread a pesticide that grows fast on the plant

(Nobori and Tsuda 2019). The good bacteria for better coffee productivity are planted growth-promoting rhizobacteria (PGPR), well-known as having a positive effect on plant growth and health stimulators (Desai et al. 2016). The PGPR can be considered the appropriate tool for agronomic practices, especially to improve beneficial bacteria around the rhizosphere.

The bacteria group of PGPR as such nitrogen-fixing, phosphate-solubilizing, and producing IAA. The previous study reported that rhizobacteria association with plants had vital benefits in the rising nutrient of plants (Hardoim et al. 2015), such as nutrient cycling and biological N-fixation (Bagyaraj et al. 2015; Caldwell et al. 2015), and enhancing plant growth (Schlaeppi and Bulgarelli 2015). Furthermore, they can produce natural metabolites that can recover soil problems caused by pesticides. Thus it can be a candidate for a natural fertilizer such as a biofertilizer (Mitter et al. 2021). However, there are significant gaps regarding fertilizer-based rhizobacteria, such as the rareness of bacteria associated with *C. canephora* and *C. arabica* in soil studies in Indonesia. The bacteria colonies around the plant root are a primary factor in maintaining plant health, increasing plant immunity, and reducing the effect of abiotic factors. The previous study on PGPR diversity associated with coffee plantation was commonly investigated by isolation in different geographical areas, such as bacteria isolated from coffee rhizosphere obtaining the *Azospirillum amazons, Asospirillum sp., Pseudomonas putida,* and *Burkholderia gladioli* that produced plant growth-promoting traits (Curi et al. 2019). However, the structure of bacteria composition in the rhizosphere by sequencing their genomics is not informed by any previous research. Understanding the percentage of bacteria presence based on molecular tools and isolation method is necessary to develop a suitable coffee production and management strategy. This information on bacteria associated with *C. canephora* and *C. arabica* rhizosphere of both methods in Indonesia is still insignificant.

Regarding the problem, the utilization of advanced molecular and conventional techniques is necessary for this research. Although, many challenges may occur due to environmental factors such as soil pH (Fierer et al. 2012), topography (Kuramae et al. 2012; Fierer et al. 2012), organic carbon number, gradient, temperature, moisture, nutrient availability (Fierer et al. 2012), irrigation water used (Dahan et al. 2014; Zhao et al. 2015), and composting effect (Chaudhry et al. 2017). Because each soil has a unique character and will perform different structure communities of bacteria (Siedt et al. 2021). Herein, this research will explore the rhizobacteria around *C. canephora* and *C. arabica* rhizosphere, investigate how bacteria profile based on their genomics, assess potential bacteria, and identify the selected bacteria containing PGPR traits. Thus, these research aims are essential to achieve the appropriate and integrated knowledge of rhizobacterial benefits for coffee production and sustainability.

MATERIALS AND METHODS

Study area and soil sampling

This study was held in Universitas Brawijaya Forest (UBF) Malang Indonesia, a specific purposive forest area. The UBF location is near the slope of Mount Arjuno, East Java, Indonesia, which is composed of pine and mahogany and other small sites that are still covered by protected forest. The local society grows coffee plantations and vegetable crops using agroforestry systems under pine or mahogany tree. The area chosen in this study is a protected forest that changed due to land clearing by the local society.

The soil samples from the rhizosphere of *Coffea canephora* (07º44'23.4' 'S and 112º32'01.2"E) at 1,135 m above sea level (m.a.s.l) and *Coffea arabica* (07º44'23.3' 'S and 11º32'01.4"E) at 987 m.a.s.l were collected from Coffee plantation (Figure 1). The sample was randomly collected using purposive sampling from three coffee plants within two meters, and the sample was collected from five coffee trees for each *Coffea* spp. Ultimately, each tree sample in triplicate was mixed to obtain a composite sample and transferred in sterilized polythene bags. The rhizosphere soil samples were stored at 4ºC for further processing in the laboratory. Physicochemical properties of the rhizosphere soil of *Coffea arabica* and *Coffea canephora* were C-organic (4.33% ± 0.7 and 5.11% ± 0.82), organic matter (8.85 ± 1.42 and 7.49 ± 1.21), total-N (0.51% ± 0.02 and 0.45% ± 0.06), C/N ratio (11.3 ± 0.57 and 8.66 ± 1.52), P-availability (3.07 ± 0.95 mg/g and 1.96 ± 0.46 mg/g) and pH (4.0 ± 0.1 and 4.5 ± 0.4), respectively.

Soil sample preparation and analysis of bacteria community

The humic acid of the soil sample was removed by the soil optimization method following Mustofa et al. (2017) with modification. Soil (0.3 g) was mixed with 300 µL of phosphate buffer (0.1 M NaH_2PO_4-Na_2HPO_4; pH 6.6) and 100 µL of 0.4 M $AlCl_3$ to equal the final volume, phosphate buffer was added to 500 µL and mixed using vortex mixer with maximum speed in 2 minutes. The suspension was treated with 1.0 M NaOH, 50 µL for *Arabica Coffea* Soil (ACS), and 90 µL for *Robusta Coffea* Soil (RCS) to increase the pH from 4 to 8. Subsequently, the tubes were inverted ten times, and the mixture was ready for DNA extraction.

Whole genomic DNA was extracted with FastDNA Spin Kit (MPBIO) following the manufacturer's protocol (https://media.mpbio.com). First, each soil suspension was added 250 µL of SDS lysis buffer (100 mM NaCl, 500 mM Tris [pH 8.0], and 10% [wt./vol]), which was mixed using vortex with the optimal speed at 10 minutes. Next, the DNA results were quantified using Nanodrop Spectrophotometer (ratio A260-280) and checked its quality through gel electrophoresis. Then, that DNA was used as a DNA template for PCR.

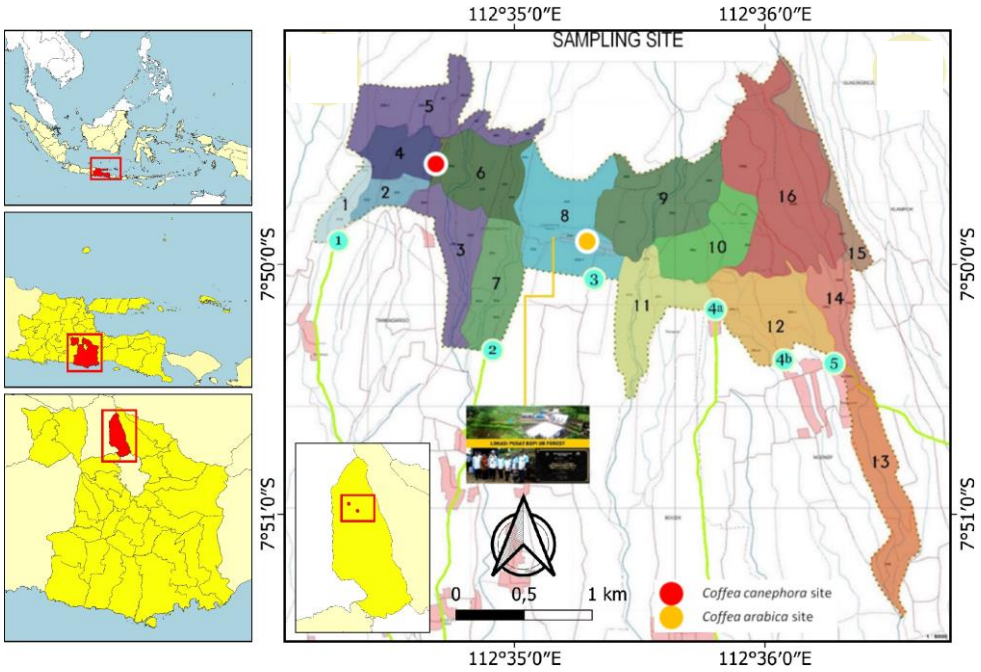


Figure 1. Sampling location from different rhizosphere soil of *Coffea canephora* (red) and *Coffea arabica* (orange) in Coffee Plantation (UB Forest), Karangploso, Malang, East Java, Indonesia

In the PCR reaction procedure, the DNA suspension 5 µL (concentration: 15 pmol/µL) was added to a 20 µL PCR mix (2x GoTaq ® Green Master Mix-Promega) and was carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs). Then, each DNA suspension was added with 2 µL (10 pmol/µL) 16S rDNA primers (515f and 806r) (Klindworth et al. 2013). The reaction was performed in 34 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 1.5 min for extension, followed by 7 min at 72°C for elongation.

Next, PCR products were purified and subjected to an automated Ilumina platform which conducted a paired-end sequencing approach of 250 bp. FLASH merged all sequence reads of raw (V1.2.7), trimmed and filtered by QIIME (V1.7.0), and clustered by Uparse (v7.0.1001) at 97% similarity. The taxonomy was assigned to OTU using the RDP classifier (Version 2.2) train on Greengenes Database. The Chao1 index and Shannon diversity were applied to determine the richness and diversity of species in the sample.

Rhizospheric bacteria isolation

Rhizospheric soil sample 25 g was dissolved in 225 mL of 0.85% sodium chloride solution, and the sample was diluted until 10^{-6}. Each aliquot sample of 0.1 mL was inoculated in Tryptic Soy Agar (TSA) with 200 µg/mL of tryptophan, Pikovskaya Agar, and N-free Bromothymol Blue (NFB) Agar based on the spread plate method to isolate and enumerate of IAA-producing bacteria (IPB), phosphate-solubilizing bacteria (PSB), and nitrogen-fixing bacteria (NFB), respectively. All PGPR bacteria were incubated at 30°C for 48 h (IPB), 72 h (PSB), and five days (NFB). All PGPR bacteria were enumerated based on the colony-forming unit method (CFU/g). Each bacteria was isolated and purified using the spread plate method and stored on a Nutrient Agar slant medium at 4°C (Israwan et al. 2015; Setia et al. 2018).

The quantification assay of rhizosphere bacteria as PGP agent

The potential of IAA-producing bacteria that release IAA hormone was determined qualitatively according to Bric et al. (1991). Each bacteria isolate was inoculated on TSA supplemented with 200 µg/mL tryptophan using the spread method. The medium surface was covered by a cellulose membrane filter (0.45 µm pore size), incubated at 30ºC for 48 hours. The membrane was removed from the plate and treated with a Salkowski Reagent. The reaction was allowed to process for 30 minutes in a dark room until pink color appeared. All isolates were tested in triplicates on separate plates and nominated the best performance of bacteria to produce IAA. All selected isolates were screened quantitatively accorded to Khalid et al. (2004) and Setia et al. (2018). Each bacteria isolate of 5 mL with equal cell density (OD=1.0) at logarithmic growth phase was inoculated into 25 mL Tryptic Soy Broth (TSB) supplemented with 200 µg/mL tryptophan, and it was incubated on a shaker incubator at 120 rpm, 28ºC for 72 h. The culture suspension was harvested at 24 h, 48 h, and 72 h and tested using Salkowsky Reagent in a dark room for 60 mins. A spectrophotometer measured the absorbance of culture suspension at 535 nm. The concentration of IAA was determined based on the IAA standard curve.

The potential of phosphate-solubilizing bacteria isolates was assayed according to Chauhan et al. (2014), Israwan et al. (2015), and Setia et al. (2018). Three loops of phosphate-solubilizing bacteria were inoculated into 25 mL Pikovskaya broth media; then, it was incubated on a shaker

incubator at 120 rpm, 28°C for 48 hours. The culture suspension of 20 µL with equal cell density was spread on the surface of the Pikovskaya agar medium and incubated at 30°C for five days. The isolates that performed clear zone were selected to be assayed quantitatively. Five milliliters of each isolate with equal cell density (OD=1.0) at the logarithmic growth phase were inoculated into 25 mL Pikovskaya broth supplemented with Ca_3PO_4 0.5%, and it was incubated at 28°C for 72 h. The phosphate concentration of the suspension culture was measured in 24 h, 48 h, and 72 h incubation time. The phosphate concentration was measured using a spectrophotometer UV-Vis at 690 nm, and the absorbance value was converted into phosphate concentration based on the phosphate standard curve.

The potential of nitrogen-fixing bacteria was screened according to Israwan et al. (2015) and Setia et al. (2018) using the Sera Test kit as primary/qualitative screening. Five loops of each isolate were inoculated into a 25 mL Nitrogen-free broth medium, and it was incubated at 120 rpm, 28ºC for seven days. The bacteria culture suspension of 2 mL with equal cell density (OD: 1.0) was harvested and centrifugated at 10,000 rpm for 15 minutes. According to the manufacturer's instructions, 2 mL of supernatant was treated using a Seta Test Kit. The selected isolates were assayed quantitatively based on ammonia production. The 5 milliliters of isolate with equal cell density (OD=1) at the logarithmic growth phase were inoculated into 25 mL NFB broth. The culture was incubated on a shaker incubator at 120 rpm, 28ºC, for seven days. Then, 3 mL of suspension was transferred into 27 mL NFB broth set at 28ºC for seven days. The bacteria suspension of 2 mL was moved into the tube, and added 10 µL $ZnSO_4$ 1 M and 2.5 µL NaOH 2N. The suspensions were incubated for 10 minutes until flocs precipitate formed and were centrifugated at 10,000 rpm for 15 mins. One milliliters supernatant was treated with 0.5 mL Nessler Reagent and 3.5 mL distilled water, and a spectrophotometer UV-Vis measured the absorbance at 425 nm. The ammonia concentration was determined based on the ammonia standard curve.

Identification of aelected PGPR bacteria based on 16S rDNA

DNA chromosome of selected PGPR bacteria was extracted using Zymo-Spin™ DNA Extraction Kit (*Quick*-DNA™ Fungal/Bacterial Miniprep Kit Catalog Number D6005). Concentration and purification of DNA were measured using nanodrop. The 16S rDNA sequence was amplified using universal primers 27f (5'-GAG AGT TTG CTG GCT ATC CAG-3') and 1492r (5'-CTA CGG CTA TGT CCT TAC GA-3') (Wang et al. 2011). The composition of 40 µL PCR Mix Solution: Nuclease-free water 11 µL, Go Taq Green Master Mix 20 µL, Primer 27f (10 pmol/ µL) 2 µL, Primer 1492r (10 pmol/ µL) 2 µL, and DNA template (15 pmol/ µL) 5 µL (Fatima et al. 2014). The amplification of 16S rDNA was carried out in PCR Mastercycle Personal (Eppendorf) with a program of denaturation at 94ºC for 5 min, followed by 34 cycles consisting of denaturation at 94°C for 30 seconds, an annealing at 55°C for 30 sec, extension at 72ºC for 1.5 min, and the final extension at 72ºC for 10 min. The amplicon of 16S rDNA was purified and sequenced in First BASE, Malaysia. The 16S rDNA sequences were aligned and analyzed by Basic Local Alignment Search Tool (BLAST) program. The phylogenetic tree was constructed using MEGA V.6 and inferred with Maximum-Likelihood using the Tamura-Nei model in 1000 replicates of bootstraps (Tamura et al. 2013).

Statistical analysis

The correlation of soil physicochemical properties and environmental factors in both soil samples was analyzed by Pearson Correlation with statistical significance ($p \leq 0.1$). Meanwhile, the potency of isolate strains production was explored using the ANOVA (two-way analysis of variance) approach. Finally, the data were analyzed for statistical significance ($p < 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All data were analyzed by IBM SPSS software 20.0. version.

RESULTS AND DISCUSSION

Physicochemical of Rhizosphere soil with the density of PGPR

Environmental factors positively and negatively affected coffee plants' PGPR density (Table 1). The rhizosphere soil from different plants showed a variety of relationships with PGPR. In this study, based on Person Correlation, soil physicochemical factors such as C/N ratio and organic matter increased the density of PGPR. Otherwise, the total-N content, the tree's diameter, water holding capacity, and altitude factors decreased PGPR density. Other factors, such as phosphate availability, reduced the density of IAA-producing and nitrogen-fixing bacteria, and soil pH increased the density of phosphate-solubilizing bacteria. For instance, IAA-producing bacteria was influenced by soil pH ($p \leq 0.05$) and tree diameter ($p \leq 0.08$); phosphate-solubilizing bacteria density was affected by P-availability ($p > 0.14$) and soil temperature ($p > 0,12$); and soil pH ($p \leq 0.07$), C/N ratio ($p \leq 0.10$) and C-organic ($p \leq 0.10$) vastly changing the density of nitrogen-fixing bacteria. Both IAA-producing bacteria and nitrogen-fixing bacteria had a significant positive relationship ($r > 0.86$), although bacteria density of IAA-producing and nitrogen-fixing had a moderately positive correlation with increasing density of phosphate-solubilizing bacteria (Table 1).

The increase in IAA-producing bacteria density showed a relationship with soil pH and tree diameter. However, the correlation analysis was performed at a moderate level. Therefore, it can be illustrated that both environmental factors changed the density of IAA-producing bacteria from *Arabica Coffea* Soil (ACS) and Robusta *Coffea* Soil (RCS) samples. A large tree diameter could produce a lot of leaves that affect the increase of litter and organic matter in the rhizosphere soil. The richness of organic matter can influence the height of litter degradation mediated by litter-degrading bacteria activity rather than IAA-producing bacteria in the coffee rhizosphere soil. Therefore, the IAA-producing bacteria decreased.

Table 1. The correlation of rhizosphere soil factors of coffee plants with PGPR density

Parameter	Bacterial density					
	IAA-producing		Phosphate-solubilizing		Nitrogen-fixing	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Soil physicochemical analysis						
Soil pH	-0.79	0.05*	0.06	0.91	-0.77	0.07*
C/N ratio	0.54	0.26	0.14	0.78	0.69	0.10*
Organic matter	0.52	0.29	0.40	0.42	0.69	0.11
C-organic (%)	-0.46	0.34	-0.56	0.24	-0.73	0.10*
Total N (%)	-0.08	0.86	-0.34	0.50	-0.03	0.94
P-availability	0.46	0.35	-0.67	0.14	0.24	0.63
Temperature	-0.49	0.31	0.69	0.12	-0.27	0.59
Water Holding Capacity	-0.14	0.78	-0.47	0.34	-0.47	0.34
Altitude (m)	-0.31	0.54	-0.08	0.86	-0.49	0.32
Light intensity (lux)	-0.35	0.49	0.06	0.90	-0.53	0.27
Tree diameter (cm)	-0.76	0.08*	-0.12	0.81	-0.74	0.08*
Tree distance (m)	0.38	0.44	-0.17	0.73	0.49	0.31
Tree height (cm)	-0.18	0.73	-0.58	0.22	-0.4	0.42
Density of PGPR bacteria						
IAA-producing bacteria	1.00	0.00	0.00	0.99	0.86	0.03*
P-solubilizing bacteria	0.00	0.99	1.00	0.00	0.41	0.41
N-fixing bacteria	0.86	0.03*	0.41	0.41	1.00	0.00
Diversity index of PGPR						
IAA-producing bacteria	-0.49	0.32	0.26	0.62	-0.51	0.31
P-solubilizing bacteria	0.49	0.32	-0.26	0.62	0.51	0.31
N-fixing bacteria	0.49	0.32	-0.26	0.62	0.51	0.31

Note: The sign (*) means the significant correlation with an alpha level of 0.1 ($p \leq 0.1$). The symbol (+) refers to the positive correlation, while the symbol (-) means the negative correlation.

Table 2. Illumina sequencing and diversity of bacteria of two coffee plantation rhizosphere

Plant	Total sequences	Taxon tags	OTU	Chao1	Shannon-Wiener	Simpson
Coffea canephora	159658	110333	2174	2.177.073	8.692	0.990
Coffea arabica	155910	108737	2225	2.282.249	8.964	0.995

Note: Unit of operational taxonomy at 97% sequence similarity based on equal subsets of all sample sequences, Chao1 is rare-based OTUs in a given sample. The highest number indicates that the species richness is height. Shannon's abundance is based on coverage, and Simpson shows species diversity based on species richness and relative abundance. Again, the highest number indicates that the species diversity is height.

Bacterial diversity in the rhizosphere coffee

The total number of operating taxonomy units (OTUs) was obtained as 314,568 sequences consisting of 159,658 and 155,910 sequences on the rhizosphere soil of Robusta coffee (RCS) and Arabica coffee (ACS), respectively. The effective sequences tag of DNA sequences was grouped based on the 97% similarity level in the OTU. The taxon number of each sample showed no significant difference, which was 110,333 taxons in RCS and 108,737 taxons in ACS (Table 2).

Thirty-four phyla detected with top-ten phyla showed a higher abundance of bacteria in each sample. The abundance of Proteobacteria dominated in both rhizosphere soil consisting of 39 % and 49.29% in ACS and RCS, respectively. Other nine phyla distributed in the different samples were *Actinobacteria* 20.76%, *Acidobacteria* 12.21%, *Chloroflexi* 3.02%, *Firmicutes* 4.85%, *Gemmatimonadetes* 2.65%, *Nitrospirae* 2.82%, *Thaumarchaeota* 1.21%, *Bacteroidetes* 1.27%, and *Verrucomicrobia* 0.69% from rhizosphere RCS sample. Meanwhile, the percentage of phyla abundance was *Actinobacteria* 24.25%, *Acidobacteria* 13.08%, *Chloroflexi* 6.91%, *Firmicutes* 5.05%, *Gemmatinonadetes* 4.77%, *Nitrospirae* 2.42%, *Thaumarchaeota* 1.61%, *Bacteroidetes* 0.5%, and *Verrucomicrobia* 0.65 % (Figure 2A).

Four classes of Proteobacteria phyla were dominated by *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria,* dan *Gammaproteobacteria* which showed ACS had 23.3% of *Alphaproteobacteria* was smaller than RCS was 32.9% of *Alphaproteobacteria* domination (Figure 2B). At the ordo level, *Alphaproteobacteria* was composed of six orders: *Rhizobiales* 82%, *Rhodospirillales* 13%, *Caulobacterales* 3.8%, *Spingomonadales* 1.1%, *Rhodobacterales* 0.8%, and *Rickettsiales* 0.1% in the RCS sample. Overall, the order of RCS and ACS was similar, except *Rhodospirillales* in ACS had an 8 % higher rather than that in RCS (Figure 2C and 2D). Following the domination of the previous order, it consisted of family dominance as *Xantobacteraceae* (Figure 2E). However, that family was dominated by *Bradyrhizobium* at the genus level (Figure 2F), and the most abundant bacteria species was *Bradyrhizobium elkanii*, 91% and 86.7% from RCS and ACS, respectively (Figure 2G).

The quantification of PGPR bacteria associated with coffee plantation

The potency of IAA-producing bacteria

Forty-five IAA-producing bacteria isolates were successfully collected, consisting of 33 ACS and 12 RCS isolates. The S1.6.3.2 RCS isolate performed the highest four coloring scores in producing IAA. Seven isolates, W1.3.4, W1.3.3, W1.4.5.3, W1.4.1, W3.3.3, W2.2.2, and W1.4.5.2, showed white color on membrane nitrocellulose which indicted the limited of IAA production. All those isolates were screened using a quantitative method to determine the IAA concentration produced by IAA-producing bacteria isolates (Table 3).

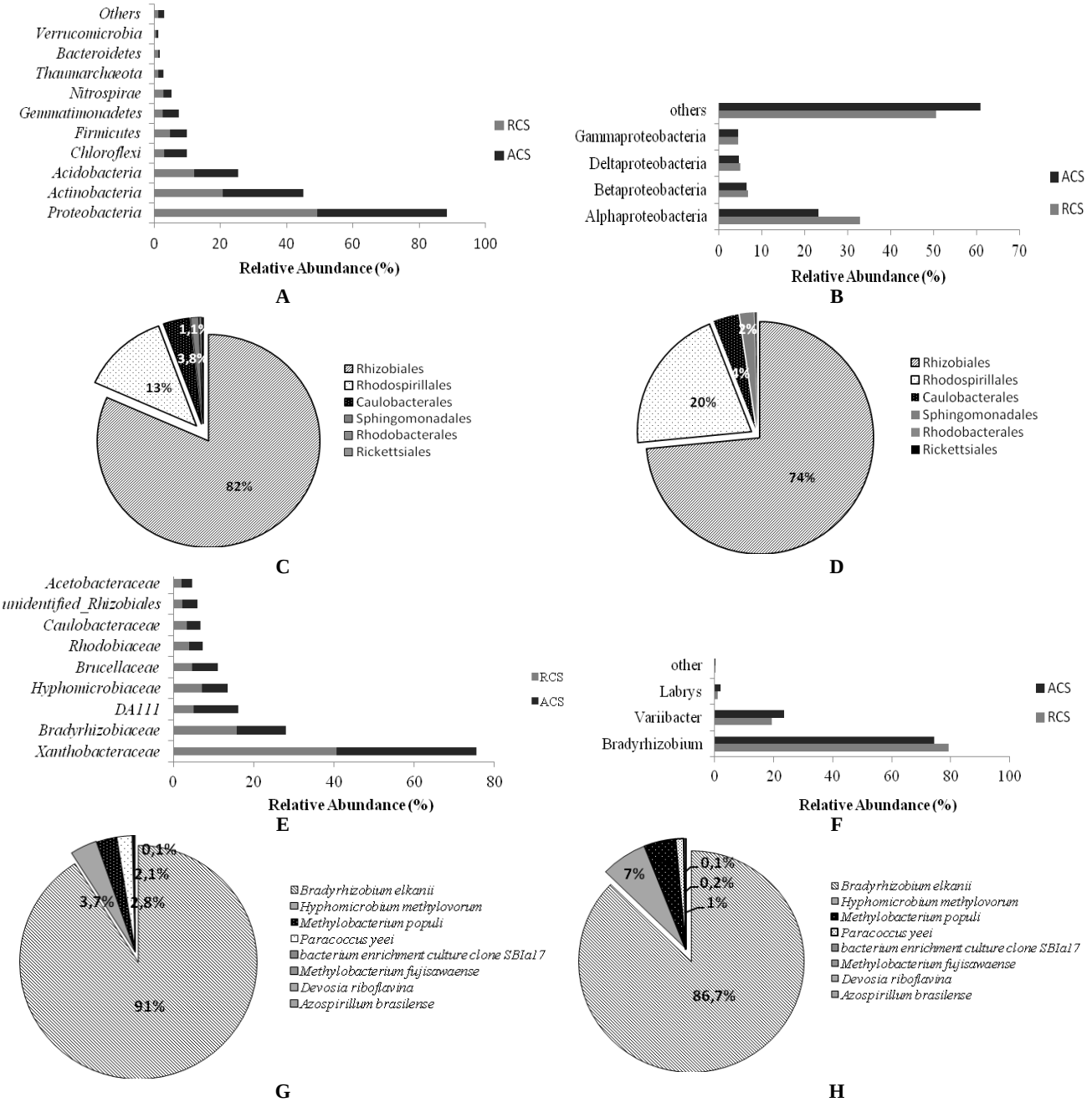


Figure 2. Relative abundance of bacterial taxa within rhizospheric bacteria communities from *Coffea canephora* (RCS) and *Coffea arabica* soil (ACS). (A) Bar plots of a ten-top taxonomic distribution of the bacterial phyla. (B) A four-class of relative abundance of Proteobacteria. (C) Percentage of a seven-ordo dominant in RCS and (D) ACS from Alphaproteobacteria. (E) The relative abundance of nine family levels of Rhizobiales. (F) The chart describes the relative abundance of three genus levels dominant in *Xanthobacteraceae.* (G) The pie shows the relative abundance of *Bradyrhizobium elkanii,* similar to the dominance of rhizospheric bacteria species from ACS (H)

Table 3. The color scoring of IAA production by rhizosphere soil bacteria of the coffee plant

Coffee plant	Isolate	Color scoring	IAA (µg/mL)	Isolate	Color scoring	IAA (µg/mL)	Isolate	Color scoring	IAA (µg/mL)
Coffea canephora	S1.4.3	2.67	83.96	S1.4	1.33	75.82	S2.3.4	3.33	84.992
	S2.2.2	2.00	45.70	S3.4.2	0.67	49.11	S1.6.2	0.67	63.24
	S2.2.3	2.00	61.11	S1.6.1	2.67	27.80	S1.6.3.1	2.67	91.45
	S1.4.1	1.67	13.94	S1.1	0.67	68.39	S1.6.3.2	4.00	104.47*
Coffea arabica	W1.6.2	0.33	22.21	W3.3.4	0.33	38.95	W2.4.4.2	1.67	88.12
	W2.2.3	0.67	25.76	W1.4.3	0.33	60.22	W2.3.4.1	0.33	48.91
	W1.3.4	0	48.87	W1.4.5.1	0.67	26.05	W1.5.2	1.67	28.35
	W2.4.3.2	1.33	84.24	W3.3.2	0.33	47.43	W3.4.2	0.67	28.81
	W2 3.1	0.33	36.89	W2.4.2	1.33	35.95	W1.4.1	0	65.17
	W3.3.1	0.33	60.67	W1.4.6	1.00	24.12	W3.3.3	0	23.53
	W3.4.3	0.67	48.74	W2.3.2	0.67	54.08	W2.4.3.1	1.67	48.91
	W1.3.3	0	15.66	W2.6.2	1.33	36.88	W3.4.1	1.00	66.36
	W1.4.5.3	0	11.13	W2.6.1.1	0.33	33.95	W2.2.2	0	31.31
	W2.2.1	0.67	46.97	W1.3.1.2	1.33	15.66	W1.4.5.2	0	15.15
	W2.6.1	0.33	16.88	W2.4.4.1	1.67	88.12*	W1.4.2	0.67	28.68

Note: The color scoring is based on membrane nitrocellulose performance after adding Reagent Salkowski. The number range from 0-0.99 (white); 1.00-1.90 (pinkish); 2.00-3.00 (reddish); and 3.1-4.00 (red). The higher the color scoring range, the more positive the IAA performance. All isolates were screened to observe the concentration of IAA production.

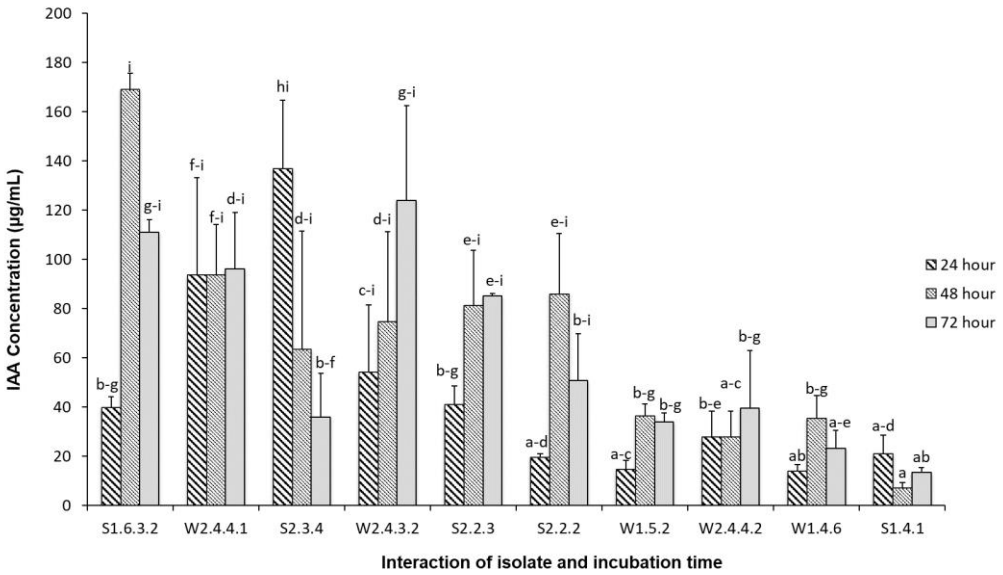


Figure 3. The production of IAA by selected IAA-producing bacteria of rhizosphere bacteria associated with *Coffea arabica* and *Coffea canephora* for three days incubation. Data are means ± SD from three replications, and values followed by a different letter (s) indicate a significant difference ($p<0.05$)

Based on the result (figure 3), each isolate differed significantly ($p<0,05$) in releasing the maximum IAA concentration at 88.12 µg/mL and 104.46 µg/mL from W2.4.4.1 of ACS and S1.6.3.2 of RCS isolates, respectively. Interestingly, after the qualitative screening, the white formation on seven isolates released the variation of IAA concentration such as W1.4.5.3 (11.13 µg/mL), W1.3.3 (15.65 µg/mL), W2.2.2 (31.30 µg/mL), W1.4.5.2 (15.14 µg/mL), W1.4.1 (65.16 µg/mL), W3.3.3(25.52 µg/mL), and W1.3.4 (48.87 µg/mL). Meanwhile, S1.4.1, S3.4.2, S1.1, and S1.6.2 of RCS isolates, white color formation, successfully released the IAA hormone at 13.94 µg/mL, 41.72 µg/mL, 68.38 µg/mL, and 63.23 µg/mL, respectively. Hence, considering the accuracy of IAA production, the quantitative method was nominated to measure the IAA concentration.

The potency of bacteria to produce IAA hormone was influenced by bacteria isolates and incubation time ($p<0.05$). For example, the W2.4.4.1 isolate had maximum production of IAA during 24 and 48-hour incubation at 93.66 µg/mL and 93.7 µg/mL ($p<0.05$), respectively, following increasing IAA concentration at 72 hours over 96.04 µg/mL ($p<0.05$). Meanwhile, the W2.4.3.2 isolate at

72 hours of incubation produced about 120 µg/mL, a higher IAA concentration than that of W2.4.4.1 (Figure 3). In other isolates from RCS samples, S2.3.4 shows the production of IAA at approximately 36.74 µg/mL at 24 hours. However, those isolated gradually decline following the next incubation time. In contrast, S1.6.3.2 was consistent with IAA-releasing, showing a great IAA concentration of over 160 µg/mL at 48 hours of incubation. Therefore, the faster and more stable producers of IAA, W2.4.4.1, and S1.6.3.2 isolates were chosen to identify based on 16S rDNA sequence similarity.

The potency of phosphate-solubilizing bacteria

In general, the 47 isolates of phosphate-solubilizing bacteria were successfully collected, including 21 ACS and 26 RCS isolates. All the isolates solubilized the phosphate by approximately 18.75%, indicated by a clear zone around the bacteria colony performing on the Pikovskaya Agar medium. The nine isolates (figure 4) consist of seven ACS isolates with solubilized phosphate of about 3.2-4.5 µg/mL and two isolates of RCS at 1.7-2.3 µg/mL. The highest phosphate-solubilizing RCS and ACS isolates were W3.5 and S131, respectively.

Based on the quantitative method, phosphate-solubilizing bacteria were influenced by the type of isolates and time of incubation ($p<0.05$) from ACS and RCS isolates. As follow, W3.5, RCS isolates could produce 7 µg/mL of the higher phosphate concentration at 24 hours of incubation ($p<0.05$) than another ACS isolate. On the other hand, almost all isolates showed a slight decline in the phosphate concentration at 48 hours of incubation. Except, RCS isolates, S131, were slightly increased at 24 and 48 hours, then dramatically dropped at 72 hours (Figure 4). Therefore, those W3.5 (ACS) and S1.3.1 (RCS) isolates were selected to identify due to their higher and faster available P concentration.

Table 4. The potency of phosphate-solubilizing bacteria from the rhizosphere soil of coffee plants

Coffee plant	Isolate	Clear zone	Phospate solubilized (µg/mL)	Isolate	Clear zone	Phospate solubilized (µg/mL)
Coffea canephora	S1.3.1	+	2.35	S2.4.2	-	0
	S1.3.2	+	2.23	S3.5.1	-	0
Coffea arabica	W1.2.4	+	1.74	W3.3.5	+	3.46
	W3	+	3.40	W3.2.3	+	3.30
	W3.5	+	4.52	W2.5.1	-	0
	W1.1.3	+	2.19	W2.3.2	-	0
	W1.2.3	+	3.70	W2.4.1	-	0

Note: The clear zone formation is an indication of phosphate-solubilizing. The positive (+) symbol means the activity of bacteria to solubilize phosphate. Otherwise, the negative (-) symbol describes the bacteria cannot solubilize phosphate

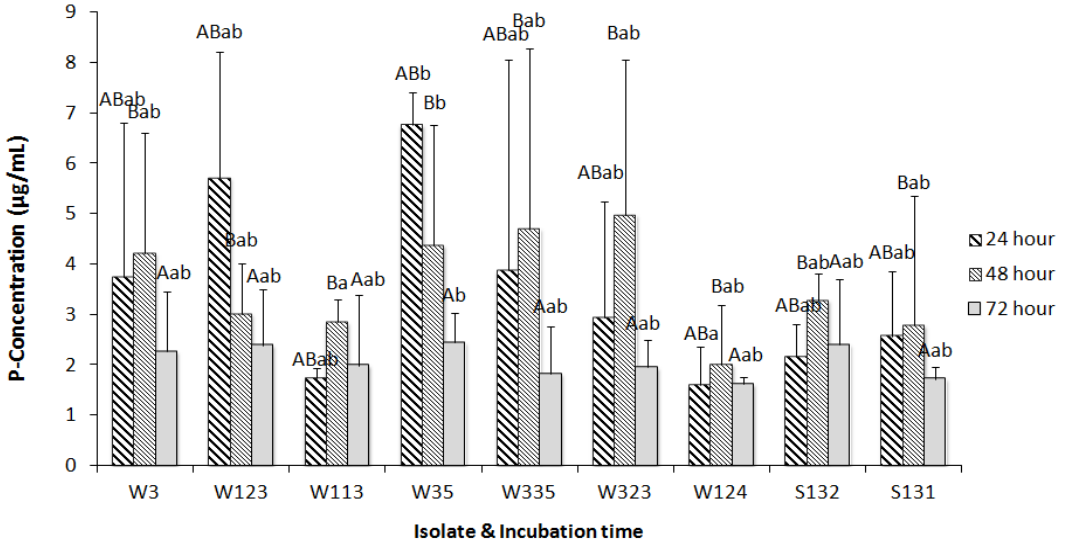


Figure 4. The production of phosphate-solubilized by selected phosphate-solubilizing bacteria of rhizosphere bacteria associated with *Coffea arabica* and *Coffea canephora* for three days incubation. Data are means ± SD from three replications, and values followed by a different letter (s) indicate a significant difference ($p<0.05$)

Table 5. The potency of nitrogen-fixing bacteria from the rhizosphere soil of coffee plants to produce ammonia

Coffee plant	Isolate	Sera test	Nessler test (µg/mL)	Isolate	Sera test	Nessler test (µg/mL)	Isolate	Sera test	Nessler test (µg/mL)
Coffea	S3.4.2	++++	2.07	S2.2.4	++++	0.95	S2.5.1	+	0
canephora	S2.2.3	+	0	S2.2.2	+++	1.08	S3.4.3	+	0
	S2.1.5.1	+	0	S3.1.1	+++	1.01	S3.4.1	+	0
Coffea arabica	W1.1.2	++	7.65	W3.4.2	++	0.11	W2.2.2	++	0.44
	W1.2	++++	21.54	W3.5.1	+++	11.44	W.3.1	+	0
	W2.1.4.1	++	0.99	W3.2.2	++	0.61	W3.1.1	+	1.63
	W2.1.5.2	+++	0.55	W3.2.1	++	0			

Note: The colorimetry of the Sera Test kit showed various color performance (+ = <0.1 mg/L (yellow); ++ = 0.1-0.4 mg/L (light green); +++ = 0.5-1.0 mg/L (green); and ++++ = > 1.0 mg/L (dark green). The best isolates of colorimetry performance are selected to screen using the Nessler method.

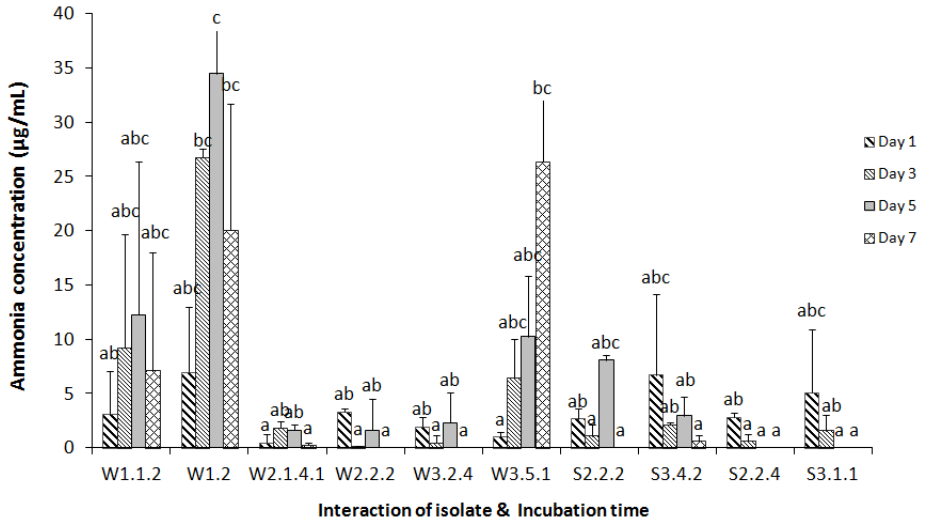


Figure 5. The ammonia production by selected nitrogen-fixing bacteria of rhizosphere bacteria associated with *Coffea arabica* and *Coffea canephora* for seven days incubation. Data are means ± SD from three replications, and values followed by a different letter (s) indicate a significant difference ($p<0.05$)

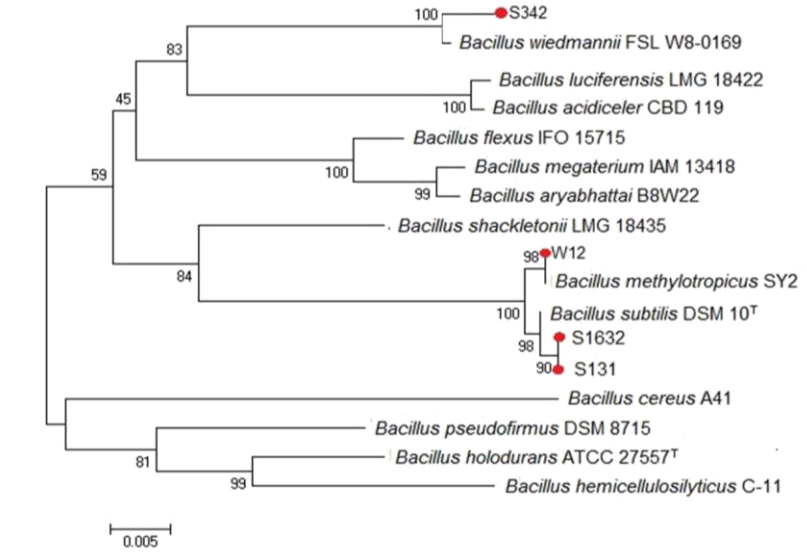
The potency of nitrogen-fixing bacteria

a sum of 21 isolates of nitrogen-fixing bacteria collected consists of 11 isolates from ACS and 10 from RCS samples. After qualitative screening using Sera Test Kit, four RCS and nine ACS isolates showed color-changing, indicating the ammonia production level was around 0.1-1.0 mg/L. Next, the higher ammonia concentration produced by those isolates was continued to screen through the Nessler method. Finally, nine ACS isolates and four RCS isolates produced ammonia approximately at 0.11-21.54 µg/mL and 0.9-2.07 µg/mL, respectively (Table 5).

Isolates W1.2 (ACS) and S3.4.2 (RCS) showed that the production of ammonia concentration was 34.48 µg/mL at 5 days and 20 µg/mL at 7 days incubation, respectively. However, almost all isolates had a declining trend of ammonia production. Only S3.4.2 and W3.5.1 showed a marginal increase over 5 days until 7 days of incubation (Figure 5). Those selected isolates were identified based on 16S rDNA sequence similarity.

Species identity of selected rhizospheric bacteria based on 16S rDNA sequence

The phylogenetic tree construction based on 16S rDNA sequences revealed that S1.6.3.2 and S1.3.1 had more than 99% similar to *Bacillus substillis* DSM 10^T (accession number: LN681568). Other isolates, S3.4.2, W1.2, W3.5, and W2.4.4.1, identified as *Bacillus wiedmannii* FSL W8-0169 (accession number: NR_152692), *Bacillus methyilotropicus* SY2 (accession number: KC790319) (Figure 6A), *Pseudomonas putida* S18 (accession number: DQ387442) (Figure 6B), and *Bacillus* spp. (phylogeny tree was not shown), respectively, with the proportion at 100% constructed from W3.5 isolates. The W2.4.4.1 phylogenetic tree was not shown due to its partial DNA sequence's lack of suitable performance. Hence, the DNA sequence was run using a Neighbour Search Tool from MOLE-BLAST with the Fast Minimum Evolution method based on MUSCLE multiple alignments to investigate its similarity at the genus level. The result showed W2.4.4.1 had low distance with Firmicutes and no access to expect with *Bacillus toyonensis* FORT24 (accession number: MG561338) and *Bacillus toyonensis* FORT 02 (accession number: MG561350) as suggested by GenBank references. Therefore, it can be predicted that the isolate was *Bacillus* spp. with a new strain.



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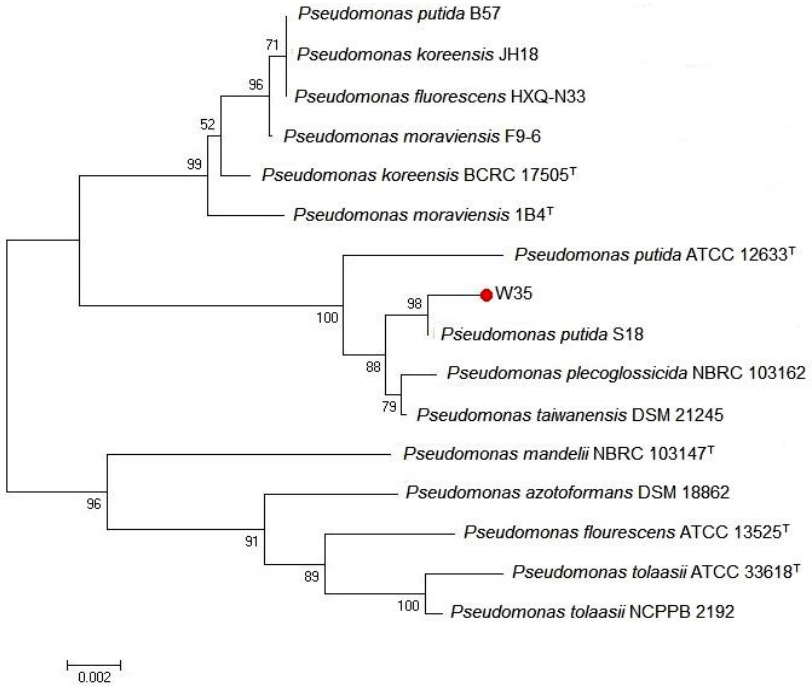

B

Figure 6. Phylogenetic trees of selected isolates (A) W1.2 (*Bacillus methylotropicus* SY2), S1.6.3.2, and S1.3.1 (*Bacillus subtilis* DSM 10^T), (B) W3.5 (*Pseudomonas putida* S18) and isolate references based on 16S rDNA sequence following *Maximum-Likelihood* with Tamura-Nei algorithm and 1,000-time bootstraps

Discussion

Correlation among physicochemical of rhizosphere soil with the density of PGPR

The low P-availability and the higher temperature increased the density of phosphate-solubilizing bacteria. The phosphate limitation in the soil triggered those bacteria to increase their activity to produce more P-solubilizing at 20°C-37°C. At that temperature, those bacteria had the optimum phosphatase enzyme to solubilize the phosphor in the soil. Otherwise, the viability of bacteria declined gradually over that 37°C. Dewanti et al. (2016) observed that the density of phosphate-solubilizing bacteria significantly decreased when incubated at 58°C caused by the bacteria activity was not capable of producing organic acid as a substantial role in phosphate solubilization.

An increasing density of nitrogen-fixing bacteria had a medium correlation with the C/N ratio, the lower pH, and the C-organic ratio. Therefore, the increase in the bacteria induces increases in the density of IAA-producing bacteria. Nitrogen-fixing bacteria promoted it to fix the nitrogen in

the soil-air to produce ammonia. Hence, ammonia was used by IAA-producing bacteria to decompose the organic matter. In the same way, Leloup et al. (2018) reported the IAA-producing bacteria were able to degrade the litter in the forest, illustrating that either N-fixing bacteria or IAA-producing bacteria activity synergized with each other. The higher the ammonia production, the more the density of IAA-producing bacteria increases. In other cases, root exudates might be affected by the growth of both bacteria densities, so the number of rhizosphere bacteria distributed in the soil around the plant root (Bhattacharyya and Jha 2012).

Soil bacterial communities in the rhizosphere of coffee plantation soil

The structure of bacteria community information from the rhizosphere coffee plantation was able to assess the soil health because the bacteria can release some secondary metabolites in those rhizospheres. The previous study (Meena et al. 2017) revealed the presence of indigenous soil microbes should be focused on exploring microbial ecology in agricultural soil. Although the comprehensive understanding of soil bacteria composition structure was crucial, it might be related to climate factors in the soil such as temperature, nutrients, moisture (Wallenstein and Hall 2012); (Shi et al. 2014), macrofauna (earthworm) (Jacquiod et al. 2020), plant diversity and micronutrient to promote plant growth and soil microbe activity in rhizosphere soil (Ghosh et al. 2017).

Both ACS and RCS samples showed Proteobacteria distribution with slightly different proportions of relative abundance at the phyla level. However, that phylum also dominated this phylum in the temperate and pine forests (Wang et al. 2018). Following the taxonomy result, the DNA sequence of bacteria discovered *Bradirhyzobium elkanii* at the species level. Those bacteria had an equal percentage proportion rate of approximately 80-90% from the samples. Commonly, *B. elkanii* had been grown around leguminous plants with mutualism symbiosis (Reeve et al. 2017).

Nevertheless, this result predicted the distribution of *B. elkanii* on RCS and ACS samples might be due to the presence of another former plant that grew on the rhizosphere coffee. Those plants interact to synergize and promote the spreading of *B. elkanii.* According to a previous study, the non-leguminous plant which showed mutual symbiosis was Fabaceae, a well-grown plant in those areas. To explore the Fabaceae plant, Rahma and Arisoesilaningsih (2017) found Kaliandra (*Calliandra perturicensis*) as a non-legume plant with similar action to the legume group to benefit each other between bacteria and plants. Hence, *C. perturicensis* was able to affect the *B. elkanii* abundance at the rhizosphere coffee plantation. Furthermore, *Bradirhyzobium elkanii* has the potential as a promoter of future natural fertilizers since their activity releases the IAA, sulfur assimilation, solubilizing-phosphate, and other components to enhance plant growth (Ahemad and Kibret 2014; Crovadore et al. 2016; Sprent et al. 2017).

The potential of PGPR bacteria associated with coffee plantation

The IAA hormone produced by IAA-producing bacteria in the ACS sample in this study at 31.04 µg/mL was higher than rhizosphere bacteria from Arabica rhizosphere in the Ethiopia forest (Muleta et al. 2013). The percentage of IAA production is more than 85% consisting of 78% and 100% from ACS and RCS, respectively. Those proportions were 9% higher than the previous study, around 80%, based on Khalid et al. (2004). The various soil bacteria species influence IAA concentration because each species has a unique capability. As follow result, each isolate produced the IAA at different times. Tallapragada et al. (2015) and Hanh TTH (2017) showed that IAA was synthesized at the stationary growth phase in maximum production at 24 hours. After 48 hours, the IAA concentration gradually declines until the next time incubation. It indicated that 24 hours was the optimum incubation time for the production of IAA.

The phosphate could solubilize the quantifying of phosphate-solubilizing bacteria under 50%, which differs from the portion result from the Muleta et al. (2013) experiment successfully dissolving around 72% by Arabika rhizosphere bacteria. Performing clear zone on Pikovskaya Agar described the bacteria stimulating phosphatase enzymes to release phosphate and calcium-binding on that growth medium (Aarab 2015). Bacteria formed the organic acid to solubilize phosphate by constructing $H_2PO_4^-$and $HPO_4^{2-}$and reducing the pH of the soil (Israwan et al. 2015); (Numan et al. 2018). According to Muleta et al. (2013), rhizosphere bacteria could be solubilized the phosphate over 40 µg/mL, while rhizosphere bacteria produced 7 µg/mL of P concentration from a sup-Alpin mountain in Himalaya, India (Pandey et al. 2006). The different concentrations of P-solubilized are influenced by acidic soil, lower P-availability, and soil type. On the other hand, it was contributed by andisol soil, containing the lower P, with a similar observation to this research by Fitriatin et al. (2017). Muleta et al. (2013) and Alcarraz Curi's (2019) method to obtain effective strains used as eco-friendly fertilizers that isolate, characterize, and determine those strains from the coffee plantation rhizosphere. The variety of ammonia concentrations is generally influenced by C-organic, which comes from coffee litter in the below-ground coffee rhizosphere soil. Those litter needed to decompose to promote the growth of the nitrogen-fixing bacteria population.

Identification of single selected PGPR based on 16S rDNA sequence

Overall, all identified isolates had multiple functions considered biofertilizer candidates. For example, *bacillus subtilis* isolated from wheat could be produced IAA hormone supplemented with L-tryptophan that releases IAA hormone at 25.5 µg/mL; to stimulate the germination and wheat seedling (Yousef 2018) and tomato (Meena et al. 2017). Another bacteria, *Bacillus wiedmannii* FSL W8-0169, was discovered by (Noha and Shixue 2018); collected from shallot and potato rhizosphere could be released IAA hormone, nitrogen-fixing, and phosphate

solubilizing to increase plant growth. Its extracellular polymeric substances (EPS) characteristic contained its surface cell, which had an essential role as mineral binding, growing soil aggregation, and biofertilizer candidate. *Bacillus methylotrophicus* of corn rhizosphere reported that it promoted shoot and root growth (Mehta et al. 2014), supplied IAA hormone for *Arabidopsis thaliana* (Pérez-Flores et al. 2017), fixed nitrogen, solubilized phosphate, and stimulated tomato growth (Ge et al. 2016). Meanwhile, *Pseudomonas putida* S18 quantified IAA, siderophore, HCN, ammonia, exopolysaccharide, and phosphate solubilization (Ahemad and Kibret 2014). Hence, all PGPR bacteria candidates could be developed as biofertilizers to apply to seed, root, or shoot of coffee plants.

In conclusion, the next-generation sequencing performed by *Bradirhyzobium elkanii* dominated all rhizosphere samples that it did not find in the culture isolation technique. In the culture method, *Bacillus* was discovered on five selected isolated bacteria. However, its abundance percentage was only below 2 %, as determined by metagenomic analysis. Nevertheless, it could quantify the particular potency related to PGPR, such as producing IAA, P-solubilizing, and N-fixing. Therefore, *Bradirhyzobium elkanii* could be analyzed for its activity as PGPR throughout an isolation technique platting on a specific medium. Subsequently, five selected bacteria, *Bacillus wiedmannii* (S342), *Bacillus methylotropicus* (W12), *Bacillus subtilis* (S131 and S1632), *Pseudomonas putida* (W35), were able to be a formulated consortium as biofertilizer. Further, those bacteria could restore soil, promote plant growth, and degrade residual pesticide components. Hence, the ecological significance of those biofertilizer candidates can improve soil health and coffee production problems.

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