Plant Growth Promoting Endophytic Bacteria of *Coffea canephora* and *Coffea arabica* L. in UB Forest

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Abstract

Plant Growth Promoting (PGP) Endophytic bacteria are used as an alternative biofertilizer to support soil health and plant productivity. This research aimed to isolate, analyze the potential, and identify the endophytic bacteria of Robusta and Arabica coffee plants as biofertilizer agents. Endophytic bacteria were isolated from the roots of coffee plants and tested for their potential to produce IAA, phosphate-solubilizing, and nitrogen fixation. Potential endophytic bacterial isolates were identified based on 16S rDNA sequence similarity. Total isolates from Robusta coffee consisting of ten IAA-producing bacteria, eight phosphate-solubilizing, and seven nitrogen fixation bacteria isolates. Total isolates from Arabica coffee roots were 12 isolates of IAA-producing bacteria, seven isolates of phosphate-solubilizing bacteria, and six isolates of nitrogen fixation bacteria. The highest potential of the isolate from Robusta roots was SS.E2 isolate to produce IAA 110.73 µg.mL^{-1}; SS.P3 isolate to dissolve phosphate 4.42 µg.mL^{-1}, and SS.N2 isolate to produce ammonium 3.15 µg.mL^{-1}. The highest potential of the isolate from Arabica roots was SW.E9 isolate to produce IAA up to 257.16 µg.mL^{-1}; SW.P5 isolate to dissolve phosphate up to 4,55 µg.mL^{-1}; and SW.N6 isolate to produce ammonium up to 1.16 µg.mL^{-1}. Isolates SS.E2, SW.E9, SS.P3, SW.P5, SS.N2, and SW.N6 were respectively identified as *Bacillus cereus* ATCC 14579, *Bacillus cereus* ATCC 14579, *Rahnella aquatilis* B35, *Kluyvera intermedia* TPY16, *Rahnella aquatilis* B35, and *Pseudomonas tolaasii* NCPPB 2192. Potential PGP isolates can be developed as biofertilizer agents for the coffee plant.

Keywords: Coffee, Endophytic bacteria, IAA, Nitrogen, Phosphate

INTRODUCTION

Coffee fruit is used as a popular beverage commodity for the global community. The value of the coffee sale price is determined by its quality. Organic coffee fruit and beverage products have a higher selling price than conventional coffee [1,2]. Robusta and Arabica coffee are agricultural commodities that commercially provide added value economically for the community and government [3].

UB Forest is a land for the conversion of forests into coffee plantations (Agroforestry). Forest land that converted to agricultural land causes a decrease in plant diversity and soil microbes [4]. Soil microbes play an important role in the cycle of elements that increase soil fertility and provide nutrients for plants [5-6]. Decreasing microbial diversity and soil fertility is a major factor in reducing the productivity of coffee plants.

Some species of bacteria have the potential to promote plant growth (Plant Growth Promoting/PGP). PGP microbes associate symbiosis that positively impacts plant health and growth, improves soil quality and nutrient cycles [7,8,9]. Endophytic bacteria associate and colonize plant tissues and play a role in spurring plant growth and development (PGP agents) [10].

Various PGP bacteria are being developed into biofertilizer products as an alternative to synthetic fertilizers. *Bacillus subtilis* endophytic cocoa beans increased the development of cocoa plants and as an antimicrobial pathogen [11]. *Bacillus subtilis* LK14 endophytic roots and stems of *Solanum lycopersicum* can produce IAA and increase root and stem biomass, as well as the amount of chlorophyll a and b [12]. *Agrobacterium tumafaciens* and *Azotobacter vinelandii* endophytic sweet potatoes produce IAA [13]. *Herbaspirillum* endophytic rice plants were able to fix nitrogen and produce IAA [14]. Endophytic bacteria from the leaves, fruits, stems, and roots of Arabica coffee plants are *Bacillus, Burkholderia, Clavibacter, Curtobacterium, Escherichia, Micrococcus, Pantoea, Pseudomonas, Serratia,* and *Stenotrophomonas* [15].

The diversity of endophytic bacteria from the roots of coffee plants are widely reported. However, the diversity and potential of endophytic bacteria in coffee plants as PGP agents, especially in UB Forest, have not been studied yet. This study aims to isolate, analyze the potential, and identify potential isolates of Robusta and Arabica coffee root endophytic bacteria from UB Forest as PGP agents.

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MATERIAL AND METHOD

Coffee Plant Root Sampling

Root samples were taken from Arabica and Robusta coffee plants in UB Forest agroforestry land, Malang, East Java Province, Indonesia. UB Forest Agroforestry is located at 07.824545°SL and 112.578390°EL and 07.821705°SL and 112.577551°EL. Three samples were taken from each type of coffee plant. Each sample was a composite root of three plants. The root sample of each plant is the secondary roots with a healthy tip at a depth ±10 cm. Root samples were put in plastic bags and stored in isothermic boxes/cool boxes.

Isolation of Endophytic Bacteria in Coffee Plants

The endophytic bacterial roots of coffee plants were isolated according to the method of previous studies [15,16,17]. The root sample of the coffee plant is cut off 10 cm long, then washed with running water and rinsed with sterile distilled water. The roots are cut into pieces with a length of ± 2 cm and then sterilized by immersing the surface in Ethanol 70% for one minute, sodium hypochlorite 5.25% for 5 minutes, and Ethanol 70% half minutes and then washed three times in sterile distilled water for one each minute. A 10 gram sterile root sample plus 90 mL of sterile physiological saline solution (0.85% NaCl) is blended to homogeneous. The root sample suspension is made in a dilution series of up to 10^{-6}. Each 0.1 mL sample suspension was inoculated in a pour plate on Tryptic Soy Agar (TSA) media containing 1 $\mu g.mL^{-1}$ L-Tryptophan and then incubated at 28°C for 48 hours to obtain a culture of IAA-producing endophytic bacterial isolates [16-17].

Phosphate solubilizing endophytic bacteria were isolated by method of previous studies [19-20]. A root sample suspension of 0.1 mL was inoculated by pour plate on a Pikovskaya agar medium consisting of Glucose (5 $g.L^{-1}$); $Ca_3(PO_4)_2$ (2.5 $g.L^{-1}$); KCl (0.1 $g.L^{-1}$); $(NH4)_2SO_4$ (0.25 $g.L^{-1}$); NaCl (0.1 $g.L^{-1}$); $MgSO_4.7H_2O$ (0.025 $g.L^{-1}$); $MnSO_4.H_2O$ (0.25 $g.L^{-1}$); $FeSO_4.7H_2O$ (0.25 $g.L^{-1}$); yeast extract (0.25 $g.L^{-1}$) and agar (15 $g.L^{-1}$), then incubated at 28°C for 72 hours.

Nitrogen-fixing endophytic bacteria were also isolated [20]. A root sample suspension of 0.1 mL was inoculated on N-free media (without Bromothymol blue) consisting KH_2PO_4 (0.5 $g.L^{-1}$); $FeCl_3.6H_2O$ (0.015 $g.L^{-1}$); $MgSO_4.7H_2O$ (0.2 $g.L^{-1}$); NaCl (0.1 $g.L^{-1}$); DL-Malic Acid (5 $g.L^{-1}$); KOH (4.8 $g.L^{-1}$), yeast extract (0.05 $g.L^{-1}$) and agar (15 $g.L^{-1}$), then incubated at 28°C for 7 days. Each IAA-producing endophytic bacterial isolate, phosphate-solubilizing, and nitrogen fixation was purified by spread plate.

Bacterial Potency Assay for Producing IAA

Each endophytic bacterial isolate was tested for its potential in producing IAA hormones [16,20]. An oose loop full isolate culture was inoculated into 25 mL of Tryptic Soy Broth (TSB) media containing 2% of L-Tryptophan (1 $\mu g.mL^{-1}$) and incubated at 28°C for 48 hours. Each cell culture with OD 1,0 as much as 5 mL was inoculated into 50 mL of TSB media containing 2% of L-Tryptophan (1 $\mu g.mL^{-1}$) and then incubated at 28°C for 72 hours. Bacterial culture was taken 2 mL every 24 hours and then centrifuged at 10.000 rpm for 10 minutes. The 2 mL supernatant was added 4 mL of Salkowski reagent and incubated in a dark room for 30 minutes until it turned pink. The suspension was measured for absorbance at a wavelength of 535 nm, then the IAA concentration is calculated based on the IAA standard curve.

Bacterial Potency Assay for Phosphate Solubilizing

Each endophytic bacterial isolate was tested for its potential in dissolving phosphate [19,20]. An oose loop full isolate culture was inoculated into 25 mL liquid Pikovskaya media (pH 7) containing 0.5% Tricalcium Phosphate (TCP) and incubated at 28°C for 72 hours. Each isolate culture (OD 1,0) was inoculated as much as 5 mL into 50 mL liquid Pikovskaya media (pH 7) containing 0.5% TCP and incubated at 28°C for 72 hours. Bacterial culture was taken 2 mL every 24 hours and then centrifuged at 10.000 rpm for 20 minutes. Supernatant 1 mL was added with 10 mL Chloromolybdate reagent and 0.1 mL Chlorostannous acid. The suspension was added with sterile distilled water up to a volume of 50 mL then incubated for 10 minutes. The sample suspension was measured for absorbance at a wavelength of 690 nm and the concentration was calculated based on a standard phosphate curve.

Bacterial Potency Assay for Nitrogen Fixation

Each endophytic bacterial isolate was tested for potential fixation of nitrogen [20]. A 1 oose loop full isolate culture was inoculated into 25 mL liquid N-Free (without Bromothymol blue) media and incubated at 28°C for 7 days. Bacterial cultures (OD 0,6) of 5 mL were inoculated into 50 mL of liquid N-Free media and incubated at 28°C for 7 days. Bacterial culture was taken 2 mL every 2 days and then added 10 µL $ZnSO_4$ and 2.5 µL NaOH 2N were incubated for 30 minutes until the

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culture became clear. The suspension is centrifuged at 10.000 rpm for 10 minutes. A 1 mL supernatant was added with 0.5 mL of Nessler's reagent and sterile distilled water up to 5 mL volume. The suspension is incubated for 30 minutes until it is yellow. The suspension was measured for absorbance at a wavelength of 425 nm and ammonium concentration was calculated based on the standard ammonium curve.

Identification of Potential Endophytic Bacteria Based on 16S rDNA Sequences

Potential endophytic bacterial isolates of PGP agents were isolated by chromosomal DNA according to the modification of the ZR Fungal/ Bacterial DNA MiniPrep Kit method. The 16S rDNA sequences were 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'). The 16S rDNA sequence was amplified with a PCR mix composition consisting of a 25 µL master mix, 5 µL DNA template (50 ng.mL^{-1}), each primer 2 µL (10 pmol), and Nuclease free water 16 µL. The PCR program for 16S rDNA amplification consisted of Pre-denaturation at 94°C (5 minutes) followed by 35 cycles including denaturation (94°C; 0.5 minutes), annealing (55°C; 0.5 minutes), extension (72°C; 1.5 minutes) and post-extension (72°C, 7 minutes) [20-21]. The 16S rDNA sequence was sequencing at First Base, Malaysia. The 16S rDNA was alignment sequence with the reference sequence, and the phylogeny tree was constructed based on the Maximum-Likelihood algorithm, with 1000 bootstraps using the MEGA 6.0 program [20,22,23].

RESULT AND DISCUSSION

IAA-producing Endophytic Bacteria

IAA-producing endophytic bacteria that were isolated from the roots of Robusta and Arabica Coffee plants were as many as 10 isolates and 12 isolates, respectively. Each endophytic bacterial isolate has a different potential ($p < 0.05$) in producing IAA hormones (Fig.1). Figure 1 shows SS.E2 isolates from endophytic roots Robusta coffee that producing IAA hormone with the highest concentration up to 110.73 µg.mL^{-1} at 72 hours incubation time ($p < 0.05$) among the endophytic bacterial isolates of Robusta Coffee plant roots. SW.E9 isolates from endophytic roots Arabica coffee were able to produce IAA hormone with the highest concentration up to 257.16 µg.mL^{-1} in 72 hours incubation time ($p < 0.05$) among the endophytic bacterial isolates of Arabica Coffee plant roots.

SS.E2 and SW.E9 isolates were able to produce higher IAA than *Bacillus aryabhattai* MBN3 endophytic *Vigna radiata* root, which produced IAA 92.03 µg.mL^{-1} [24]. The L-tryptophan compound as an IAA precursor that was added to the media can increase the production of IAA, and bacterial culture in the media will trigger auxin biosynthesis (IAA) [25]. The addition of L-tryptophan as much as 0.2 mg.mL^{-1} produced the highest IAA of 62.92 µg.mL^{-1} in SB28 isolates [18].

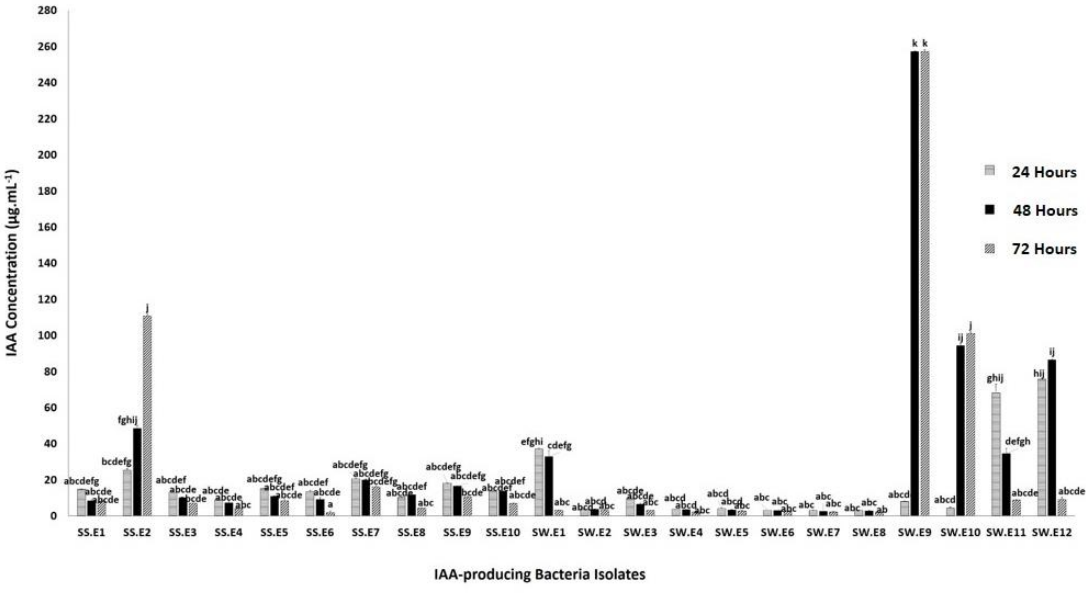

Figure 1. The concentration of IAA hormone produced by IAA-producing bacteria at various times incubation.
*Data were expressed as mean ± standard deviation of three replications using Two-Way ANOVA analysis at α = 0.05. The notation above of the different histograms states the difference in potential between isolates ($p < 0.05$).

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Phosphate Solubilizing Endophytic Bacteria

Phosphate Solubilizing Endophytic Bacteria from roots of Robusta Coffee and Arabica Coffee plants were successfully isolated as many as 8 isolates and 7 isolates, respectively. Each isolate can dissolve different phosphates (Fig. 2). Figure 2 shows the isolates of SS.P3 from endophytic roots Robusta Coffee plants in 48 hours incubation time had the highest potential ($p<0.05$) to dissolving phosphate up to 4.42 $\mu g.mL^{-1}$. SW.P5 isolates from endophytic roots Arabica Coffee plant has the highest potential ($p<0.05$) dissolving phosphate with a concentration up to 4.55 $\mu g.mL^{-1}$ at 48 hours incubation time.

The concentration of SS.P3 and SW.P5 isolates were lower than EB14 isolates that can dissolve phosphate for 12.54 $\mu g.mL^{-1}$ with an incubation time of 48 hours [26]. In general, bacteria can dissolve phosphate because it produces organic acids, which reduce the pH of the media [27]. By using tricalcium phosphate (TCP) as a P source in the culture medium, produced the highest phosphate concentration reaching 764.7 $\mu g.mL^{-1}$ [28].

Nitrogen-Fixing Endophytic Bacteria

A total of 7 isolates and 6 isolates of nitrogen-fixing endophytic bacteria were found from the roots of Robusta Coffee and Arabica Coffee, respectively. Each isolate can produce different ammonium (Fig.3). Figure 3 shows that SS.N2 isolate from endophytic roots Robusta Coffee plants has the highest potential ($p <0.05$) producing ammonium up to 3.15 $\mu g.mL^{-1}$ at the incubation time of 3 days (72 hours). The highest ammonium concentration from endophytic roots Arabica Coffee plant is SW.N6 isolate that reached 1.16 $\mu g.mL^{-1}$ at 7 days incubation time ($p <0.05$). The potential of SS.N2 and SW.N6 isolates was almost the same as EB5 isolates in producing 1.6 $\mu g.mL^{-1}$ ammonium [26]. Molecular nitrogen is modified by endophytic bacteria to be converted into ammonium as a nutrient for the growth of host plants [29].

Potential Endophytic Bacterial Species of PGP Agents

PGP activity by isolates, which indicated the highest IAA production, was identified as *Bacillus cereus* ATCC 14579^T SS.E2 and SW.E9 isolate. The isolates that had the highest phosphate-solubilizing concentration was identified as *Rahnella aquatilis* B35 isolate SS.P3 and *Kluyvera intermedia* TPY16 isolate SW.P5. The highest ammonia production activity was performed by *Rahnella aquatilis* B35 isolate SS.N2 and *Pseudomonas tolaasii* NCPPB 2192 isolate SW.N6. The potential isolates have each different potency in their activity as PGP. Each plant has endophytic bacteria that capable of producing biological compounds or secondary metabolites obtained from the transfer host plant to endophytic bacteria [30].

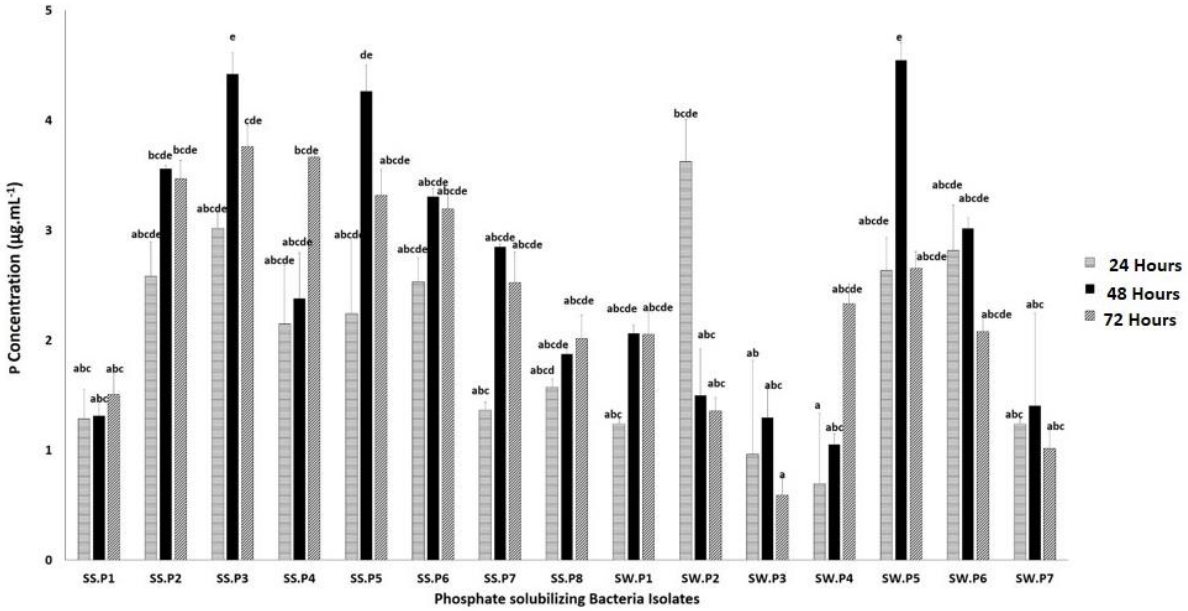

Figure 2. The concentration of Phosphate dissolved by Phosphate solubilizing bacteria at various times incubation.
*Data were expressed as mean ± standard deviation of three replications using Two-Way ANOVA analysis at α = 0.05. The notation above of the different histograms states the difference in potential between isolates ($p <0.05$).

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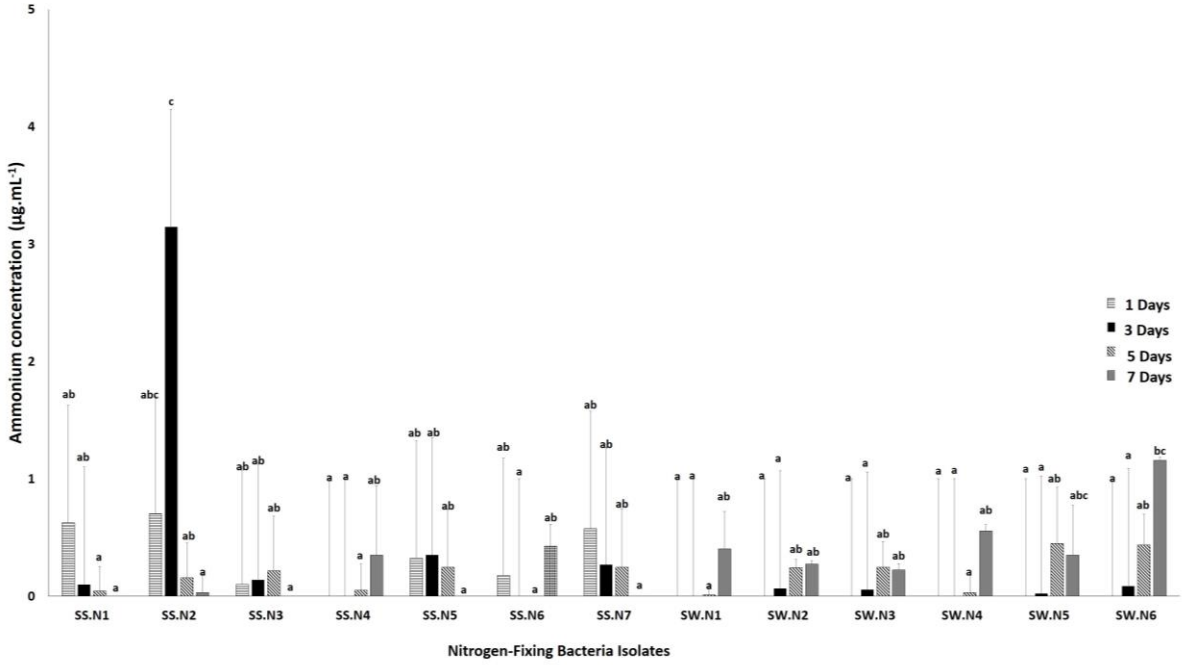

Figure 3. The concentration of Ammonium produced by Nitrogen-Fixing bacteria at various times incubation
*Data were expressed as mean ± standard deviation of three replications using Two-Way ANOVA analysis at α = 0.05. The notation above of the different histograms states the difference in potential between isolates (p <0.05).

Phylogenetic tree of Potential Bacteria Species Based on 16S rDNA

The phylogeny tree of selected IAA production isolates (SS.E2 and SW.E9) were constructed from 16s rDNA sequences and compared with reference strain sequences. As shown in figure 4a, isolates SS.E2 and SW.E9 are in the same cluster as *Bacillus cereus* ATCC 14579^T.

The SS.E2 and SW.E9 isolates have sequence similarity (99.9%) with *Bacillus cereus* ATCC 14579^T. The SS.P3 and SW.P5 isolates were identified as *Rahnella aquatilis* B35 and *Kluyvera intermedia* TPY16 with similarity values of 99.9%, respectively (Fig. 4b and 4c). The SS.N2 and SW.N6 isolate was identified as *Rahnella aquatilis* B35 and *Pseudomonas tolaasii* NCPPB 2192 with similarity values 99.9% and 99.0%, respectively (Fig. 4c and 4d).

The genus *Pseudomonas* and *Bacillus* are commonly found as endophytic bacteria. Plant growth promoting MQ23 and MQ23R endophytic bacteria were identified as *Bacillus cereus* ATCC 14579^T (100%), had the N_2 binding gene (*nifH* gene) and were able to produce siderophore, IAA, and antifungal activity [31]. *Bacillus cereus* ATCC 14579 and *Bacillus aerius* 24K strains are known for producing IAA and ACC-Deaminase activities, respectively [32]. Endophytic *Bacillus subtilis* strains had PGP activity such as phosphate solubilizing, IAA production, and biological nitrogen fixation, which can significantly increase the dried weight of the aerial part, the dried weight of the radicular system, the diameter of the stem, and the number of leaves in eucalyptus plants [33]. *Pseudomonas taiwanensis* and *Pseudomonas geniculata* strains have PGP activities such as ammonia production, HCN, IAA, siderophore, phosphate solubilizing, and ACC deaminase activity [34].

Rahnella sp. are positive reported the N2 binding gene (nifH gene) based on PCR amplified by *nifH* and *nifH-b1* primers [35]. *Rahnella aquatilis* from the soybean rhizosphere had phosphate solubilizing activities with the organic acid release that drop the pH of the culture medium [36]. The *Kluyvera* genus as a PGP agent has not been much explored and reported. However, the study found that *Kluyvera ascorbata* could be used to control *Plutella xylostella* (Lepidoptera: Plutellidae) [37].

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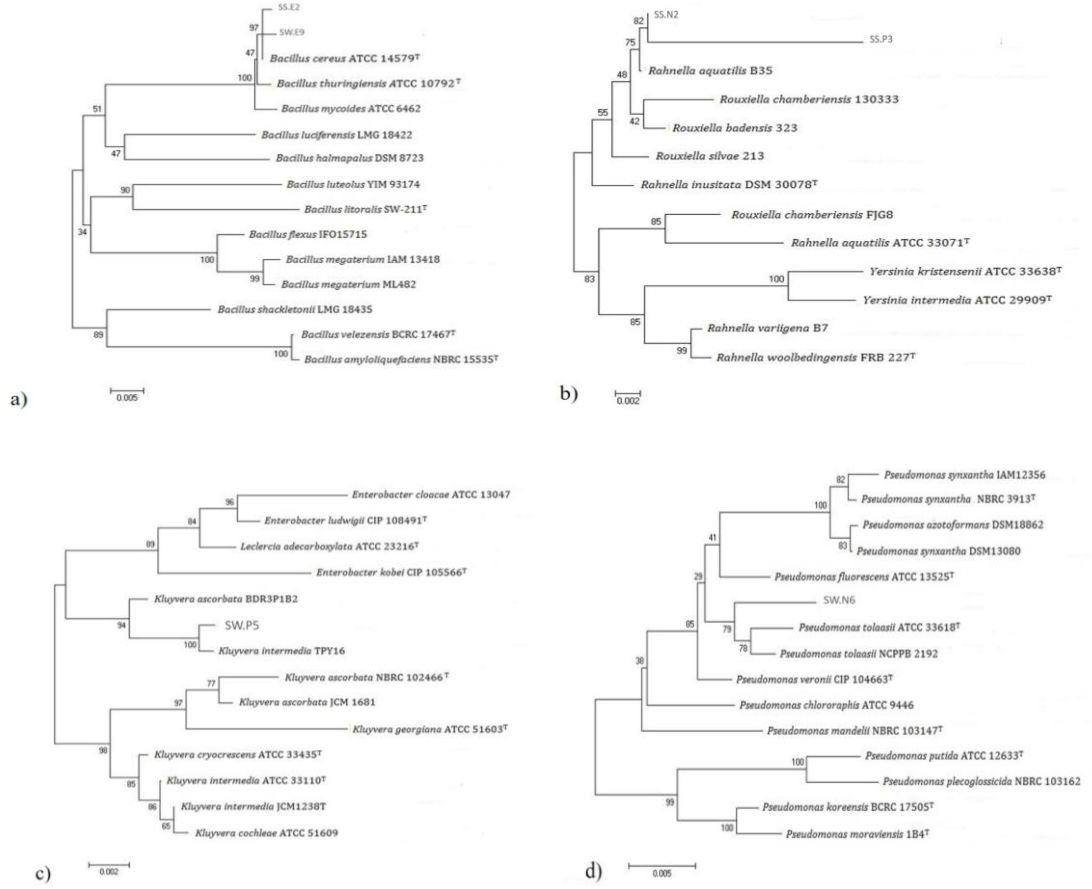

Figure 4. Phylogenetic Tree of Potential Bacteria as PGP using Maximum-Likelihood algorithm with 1000 of bootsraps, MEGA 6.0 program: a) SS.E2 & SW.E9 isolate, b) SS.P3 & SS.N2 isolate, c) SW.P5 isolate, d) SW.N6 isolate

CONCLUSIONS

Based on the results of the study, we concluded that isolates endophytic root Robusta coffe and Arabica coffe can be developed as PGP agents. SS.E2 and SW.E9 isolates produced the highest IAA were respectively identified as *Bacillus cereus* ATCC 14579 (99.9%). SS.P3 and SW.P5 isolates is the highest to dissolving phosphate that respectively identified as *Rahnella aquatilis* B35 (99.9%) and *Kluyvera intermedia* TPY16 (99.9%). Meanwhile, Isolates SS.N2 and SW.N6 is the highest to fixing nitrogen and respectively identified as *Rahnella aquatilis* B35 (99.9%), and *Pseudomonas tolaasii* NCPPB 2192 (99.0%).

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