Isolation and Identification of Nitrogen-Fixing Rhizobacteria associated with Cocoa plantation (*Theobroma cacao* L) as Biofertilizer Agent

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Abstract

Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency, is one of the centers for cocoa plantations using an organic farming system. However, over time this organic farming system experienced a decrease in fruit production, possibly from soil fertility and biofertilizers that were less available in the soil. This study aims to analyze the nitrogen-fixing ability and identify rhizosphere isolates that excel in nitrogen-fixing obtained from the rhizosphere of cacao (*Theobroma cacao* L) plant. Bacteria were isolated from the soil surrounding cocoa plant roots and grown on Nfb (*Nitrogen free Bromothymol Blue*) agar media. The nitrogen-fixing bacteria were analyzed with quantitative and qualitative methods. Six potential nitrogen-fixing isolates were identified based on the 16S rDNA sequence. The total number of isolates obtained from nitrogen-fixing isolation was six isolates. The R3.FN1 isolate showed the highest ammonia index at 0.52 µg.L^{-1} and was identified as *Stenotrophomonas maltophilia* KB13 with 99.87% similarity to the 16S rDNA sequence.

Keywords: Cocoa, Nitrogen-fixing**,** Rhizosphere, *Stenotrophomonas maltophilia.*

INTRODUCTION

The cocoa plant (*Theobroma cacao* L.) is a member of the Sterculiaceae family of the *Theobroma* genus, originating from the Amazon and other tropical areas in Central and South America. In Indonesia, the cocoa plant was first introduced by the Spanish around the XV century [1]. Indonesia's cocoa bean production is produced by 95% from independent smallholdings, 3% by Private Large Plantations, and 2% from State Large Plantations [2]. Indonesia is the 3rd largest cocoa bean-producing country in the world, after Ghana and Côte d'Ivoire. Indonesia accounted for 14% of the world's cocoa bean production in 2017, 657.000 tons from a total of 4.744.000 tons [3]. Most of the residents of Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency work as planters. Some of the commodities that are cultivated on farmers' land are sugar cane, cocoa, and coffee. In the history of Ringinkembar Village, the potential as the largest cocoa producer at that time was in Sumbermanjing Wetan District, with an area of more than 1000 hectares. However, over time it decreased to 125 hectares [4].

Biofertilizers are more environmentally friendly in promoting plant growth than synthetic fertilizers commonly used. Previous studies have shown that biofertilizers increase yields by 20-30% and can replace synthetic N and P fertilizers by 25%. Plant growth promoter rhizobacteria (PGPR) is one of the most widely used biofertilizers in agriculture. PGPR agents that play a role include *Azotobacter* sp., *Pseudomonas* sp., and *Bacillus* sp. [5]. Biofertilizer is a fertilizer that contains microbes, and these microbes help provide nutrients in the soil so that they can be used directly by plants [6]. The periodic provision of biofertilizers can help the plant's immune system by improving the balance of nutrients through the soil so that plants become more productive.

Efforts to obtain plant growth promoter bacteria from the rhizosphere of cocoa plant in Indonesia have not been done much. This research was conducted to obtain rhizosphere bacteria that can fix nitrogen. These bacteria were isolated from cocoa plantation soil in Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency, as a biofertilizer to replace synthetic pesticides or chemical fertilizers.

MATERIAL AND METHOD

Rhizosphere Soil Sampling

The sampling location is one of the organically managed cocoa plantations in Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency. Three selected plants with coordinate locations, plant 1: S"08°16'14.3" E"112°43'.0"; plant 2: S"08°16'15.0" E"112°43'42.9" and plant 3: S"08°16'14.5" E"112°43'43.2". Next, for each plant, samples of

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the rhizosphere soil were taken from five points. The five samples were composited. Thus, a total of three rhizosphere soil samples were obtained. Samples were stored in an isothermic box at 4-10° C during the trip to the laboratory.

Isolation of nitrogen-fixing bacteria

Twenty five grams of rhizosphere soil sample was suspended in 225 mL of 0.85% NaCl solution to obtain a dilution of 10^{-1}. The next step is to make a dilution series of 10^{-2} to 10^{-6} in 9 mL of 0.85% NaCl solution. The suspension of each dilution was taken at 0.1 mL and inoculated using the *spread plate* method on Nfb Agar media. With the composition ($g.L^{-1}$): KH_2PO_4 0.5; $FeCl_3.6H_2O$ 0.015; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; DL-Malic Acid 5; KOH 4.8; Yeast exstract 0.05; Agar 15 in 1 liter aqua dest [7].

The cultures were incubated at 28°C for seven days. All bacterial colonies are considered nitrogen-fixing bacteria. Bacterial cell density per gram of sample was calculated by TPC (*Total Plate Count*). The different colony morphology was characterized, and the bacterial diversity index was calculated using the Simpson diversity index. The bacteria were then purified using the spread plate method and confirmed their purity by Gram staining. The single colonies obtained were stored as culture stock in NA medium, which was incubated at 28°C for 24 hours [8].

Quantification of nitigen fixing rhizobacteria

Twenty-one isolates were quantified using the qualitative method. Qualitative tests were carried out by inoculating two loops of culture suspension into 5 mL of semi-solid NFb medium by adding 0.3% agar concentration in a test tube. The pellicle was shown, or color changed from green to blue within seven days at 28°C, indicating the activity of nitrogen-fixing rhizobacteria.

Six isolates were tested with a quantitative method using Sera Ammonia Test Kit with a modified procedure. The test was conducted based on a factorial Completely Randomized Design (CRD) with three replications. The independent variables used were the type of isolate that passed the qualitative test screening and incubation time. Five mL culture starter of each isolate 10^7 cells.mL^{-1} were grown in 80 mL of liquid NFb medium without the addition of Bromothymol Blue. Medium without the addition of inoculum was used as a control. Control and test cultures were statically incubated at 28°C for seven days. Ammonia produced by each isolate was measured at incubation at 0, 2, 4, and 8 days. Cultures were taken 5 mL and centrifuged at 6.000 rpm, 10°C, for 10 minutes. Total of 2.5 mL supernatant was diluted with the addition of 2.5 mL of distilled water. The reagents provided by the Sera Ammonia Test Kit were added. An indication of the nitrogen-fixing activity of each isolate was a change in the color of the medium from clear to green or blue. The results obtained were quantified by spectrophotometry at a wavelength 700 nm. Ammonia concentration was calculated based on the standard of ammonia curve equation [9]. The nitrogen-fixing parameter data were analyzed for variance using one-way ANOVA ($p \leq 0.05$). If there is a significant difference, then proceed with the Tukey test.

Identification of Bacteria Based on 16S rDNA

The selected isolate were extracted for their chromosomal DNA according to the *Zymo Research* protocol, Quick-DNATM Fungal/Bacterial Miniprep Kit, USA. Bacterial 16S rDNA sequences were amplified using a Thermocycler Amplitron® with universal primers 27f (5' AGA GTT TGA TCC TGG CTC AG) and 1492r (5' GGT TAC CTT GTT ACG ACT T) with the PCR program: Pre-Denaturation 94°C for 5 minutes, Denaturation 94°C for 30 seconds, Annealing 52°C for 30 seconds, Extension 72°C for 1.5 minutes and final extension 72°C for 10 minutes.

Amplicons were sequenced using the Automatic Sequencer Analyzer ABI 3130, which was done by Firstbase Malaysia. The 16S rDNA sequences were edited using the Sequencer Scanner V.1 program and combined using the CAP Contig Assembly in the BioEdit V.7.2 program. 16S rDNA sequences were BLAST using the NCBI BLASTN program, and nucleotide sequences were determined according to *GenBank* [10]. The isolated 16S rDNA sequences and the reference strain were aligned with the ClustalW Multiple Alignment program MEGA 11. The phylogeny tree was constructed and inferred using the *Nearbor-Joining* (NJ) algorithm, using 1000 times bootstrap [11].

RESULTS AND DISCUSSION

The average nitrogen-fixing bacteria density of the three samples was 1.40×10^4 $cfu.g^{-1}$. According to the result, N-fixing rhizobacteria was influenced by C-organic, N-total, C/N ratio and organic matter. The area around the roots whose chemical, physical and biological properties are influenced by root activity [12]. Chemical parameters of the rhizosphere soil of cocoa plants showed C-organic content of 4.71 ± 0.07%. This value is classified as high.

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Table 1. Physicochemical parameters of cocoa plant soil

Parameter	Average	Description
Organic matter (%)	8.74 ± 0.04	
C- Organic (%)	4.71 ± 0.07	High (3-5)
N- Total (%)	0.44 ± 0.61	Medium (0.21-0.50)
P- Total (mg.kg^{-1})	565.24 ± 0.43	
C/N ratio	10.70	Low (<11)
pH	5.72 ± 0.80	
Air temperature (°C)	25 ± 0	
Light intensity (lux)	2171 ± 1018	
Water content (%)	28.59 ± 0.26	
Bacterial density		
Nitrogen-fixing bacteria (cfu.g^{-1})	1.40 x 10^4	
Diversity Index		
Nitrogen-fixing bacteria	0.50	Medium (0.31-0.60) [13]

The content of organic matter is 8.74 ± 0.04%, which is classified as very high. The nitrogen content (N-total) of 0.44 ± 0.61% was classified as moderate. Based on these data (Table 1), it is known that the organic carbon content in the rhizosphere soil of cocoa plants is higher than the nitrogen content.

The high levels of C-organic in the soil are influenced by high organic matter. The higher the levels of organic matter, the higher the amount of nitrogen it contains so that plant growth will be good. However, if the C-organic is too high, it will affect the life of the organism population and cause a shading effect [14]. Based on the criteria for assessing the chemical properties of the soil research center [15], this research plot was classified as having a moderate total N. It is directly proportional to the organic matter content, where the higher the soil organic matter content, the higher the total N-content of the soil. In other words, every change in organic matter content will change the total N-content. The availability of N in the soil is not only determined by the amount of N-total soil. It is also closely related to the organic matter content of the soil, especially the level of decomposition (C/N) [16,17]. The rhizosphere C/N ratio of cocoa plants was 10.70 in this study, and the C/N ratio of the soil type of cocoa was low. It is because some of the available N is used by microorganisms in the breakdown of organic matter [18].

Bacterial density was influenced by high C-organic, moderate total nitrogen, low C/N ratio, and high organic matter in the rhizosphere samples of cocoa plants (Table 1). Organic matter is essential in the density and pH of microorganisms [19]. The low pH in cocoa plants is caused by the activity of fermenting microorganisms to form organic acids (acetic acid, pyruvic acid, and lactic acid) [20,21]. C and N are macromolecules with structural and functional roles in bacterial cell components [22].

Potential Isolates of Nitrogen-Fixing Bacteria

The results of the qualitative potency test of 21 isolates showed a change in the color of the medium. Based on the qualitative test, it was found that 51% of the total isolates showed a color change in semi-solid NFB media, from green to bluish, six from plant 1, seven from plant 2, and eight from plant 3. The presence of pellicle produced by bacteria in the medium is caused by the absence of excess oxygen in the medium. The rate of oxygen diffusion is in line with the respiration rate of the organism, which is a good condition for the activity of the nitrogenase enzyme, which assisted in the reduction of acetylene to ethylene [23]. The color changed in the medium from green to blue with bromthymol blue as a color indicator. Bromthymol blue changes color if there is an increased pH in the medium due to nitrogenase activity [24]. The medium was able to provide the nutrients needed by nitrogen-fixing bacteria, a blue color change in the medium indicated that there was nitrogenase activity carried out by nitrogen-fixing bacteria [25].

The quantitative nitrogen fixation potential test (Fig. 1) showed the difference in ammonia concentration of each isolate was influenced by different types of isolates and variations in incubation time ($p<0.05$). R3.FN1 has the highest ammonia concentration with 0.52 mg.L^{-1} at two-day incubation. This isolate was able to produce ammonia for up to eight days of incubation but decreased up to 0.22 mg.L^{-1} because the ammonia was used for bacteria [26]. It was found that all isolates experienced a decrease in ammonia concentration at four and eight days of incubation.

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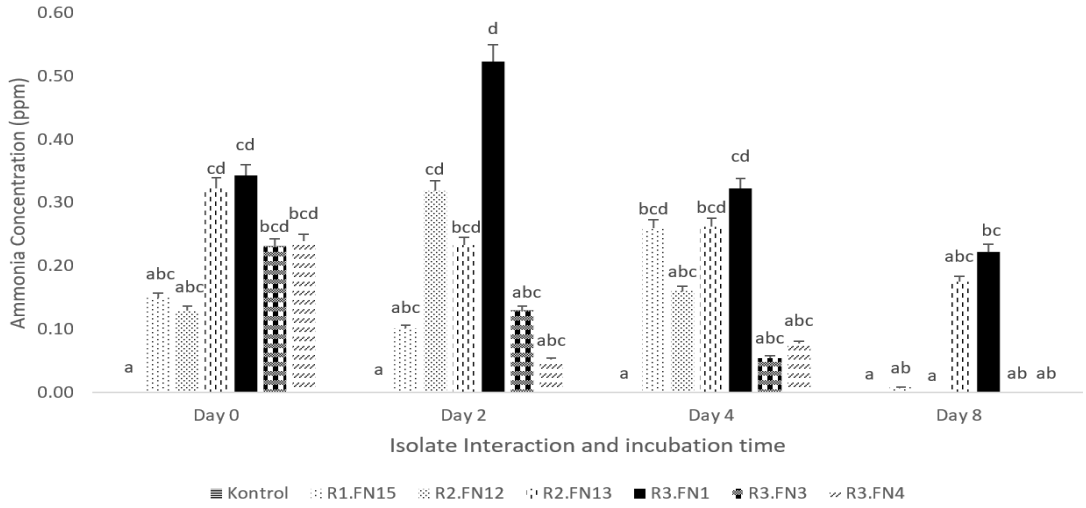

Figure 1. Ammonia concentration from nitrogen fixation activity of rhizosphere bacterial isolates of Cocoa plants with variations in incubation time
*The different notations above the histogram show significant differences between isolates and between isolates' incubation time ($p<0.05$)

The variation in ammonia concentration was due to the different abilities of each isolate to fix nitrogen into ammonia. It was due to the different types of bacterial isolates [27]. Nitrogen-fixing bacteria convert N2 in the air into ammonia using the enzyme nitrogenase. Based on the ability to produce ammonia with stable and highest criteria, the isolate R3.FN1 was selected to be identified.

Molecular Identification of Nitrogen-Fixing Bacteria

Potential nitrogen-fixing bacteria R3.PF1 isolates from Ringinkembar Village were identified phylogenetically based on the similarity of the 16S rDNA sequence. Figure 2 shows that the isolate R3.FN1 was identified as *Stenotrophomonas maltophilia* KB13 with 99.8% similarity to the 16S rDNA sequence (Fig. 2).

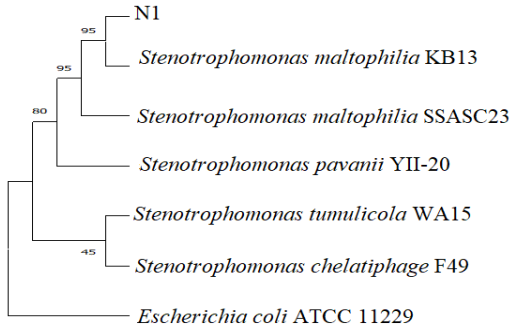

Figure 2. The phylogeny tree showed the relationship of R3.FN1 isolates and references bacterial strains based on 16S rDNA sequence according to the *Neighbor-joining* algorithm

Stenotrophomonas maltophilia is a bacillus, gram-negative, non-fermenting, non-sporulating bacterium with polar flagella 0.5-1.5 in length [28]. It is often found in the rhizosphere or the soil around plant roots. These bacteria can be found widely in the natural environment [29]. The genus *Stenotrophomonas* is the dominant genus in nature (water, soil, and plants), has a wide distribution, and plays an important role in the nitrogen and sulfur cycle [30].

CONCLUSIONS

The rhizosphere isolate of cocoa plants were taken from Ringinkember Sumbermanjing Village, Malang Regency had the highest potential as a nitrogen-fixing activity of 0.52 $mg.L^{-1}$. The isolate R3.FN1 was identified as *Stenotrophomonas macrophilia* KB13 with 99.87% similarity. Therefore, the *S. maltophilia* is a potential bacteria for PGPR and plays an important role in the nitrogen cycle to be used for Cocoa plants.

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