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Exploration and Identification of Rhizobacterial Morphology in Cocoa Plantations of Central Sulawesi, Indonesia

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Abstract: Indonesia is the third largest producer of cocoa beans globally, following Ghana and the Ivory Coast. Its current annual production stands at 600,000 tons. Despite these statistics, the cocoa's quality remains relatively subpar compared to its potential output, as exemplified by the Sulawesi III clone, which has the capacity to produce 2 to 2.4 tons per hectare. This research aims to isolate indigenous bacteria from Meko village so as to examine their distinct functions in improving the health and productivity of cocoa plants. The research method consisted of gathering soil samples from cocoa farms followed by bacterial isolation using Luria-Bertani (LB) culture. Soil samples were collected from the cocoa plantations in Meko Village, specifically targeting areas adjacent to cover crops, bordering woods, near sugar palm trees, and isolated locations. The soil was suspended in sterile water and serially diluted up to a concentration of 10^-6. Each dilution was cultured on Luria-Bertani Agar (LBA) plates and incubated for four days. Isolates were subsequently selected based on distinct morphological differences observed under a stereomicroscope. A total of 25 bacterial isolates were characterized, revealing a wide range of morphologies with variations in shapes (round, irregular), surface textures (rough, dull, slimy), appearances (opaque, cloudy, colored with yellow and reddish hues), and elevations (flat, rising). Most isolates had a rod-shaped morphology, with 23 of them classified as gram-negative and three as gram-positive. Out of the total isolates, 19 of them had a rod-shaped cellular morphology while the remaining six had a spherical one.

Keywords: *Bacteria, colony morphology, gram stain, and rhizobacteria*

1. Introduction

Theobroma cacao L. is the tropical plant requiring approximately 70% protection from sunshine, which is optimal for its growth. Due to its tropical climate, Indonesia provides favorable conditions for the cultivation of cocoa plants. Because of its shade requirements, this plant is well-suited for plantations that have a diverse range of plant species. Cocoa, being one of the commercially produced species, is particularly attractive for marketing purposes. Indonesia is the third largest producer of cocoa beans globally, following Ghana and the Ivory Coast.

The current annual cocoa bean production in Indonesia stands at 600,000 tons. Therefore, cocoa is one of the primary export commodities in Indonesia. Despite such ranking, its manufacturing capacity remains the third highest globally, trailing Ivory Coast and Ghana. Indonesia possesses cocoa plantations spanning a total area of 1,592,562 hectares. These plantations consist of 297,711 hectares of immature plantings, 248,013 hectares of damaged plantations, and exhibit an average productivity of 740 kg per hectare. Central Sulawesi is one of the provinces in Indonesia that has the highest cocoa plantation area. The total land area dedicated to cocoa cultivation in this province is 282,773 hectares, with a productivity rate of 687 kilograms per hectare. Therefore, cocoa is a major agricultural product grown in Central Sulawesi. Nevertheless, the overall production remains lower compared to North Sumatra Province, which boasts a productivity rate of 987 kg per hectare (Statistics Indonesia, 2021).

The quality of cocoa remains relatively low in comparison to its potential harvest, as exemplified by the Sulawesi III clone, which has the capacity to produce 2 to 2.4 tons per hectare. The problems arising from soil damage in cocoa plantations are primarily caused by factors, including the excessive use of pesticides. Additionally, soil degradation occurs causing decreased levels of organic matter and nutrients due to harvesting and washing processes (Ling *et al*., 2014). The suboptimal production of cocoa plantations in Indonesia is partly due to the inferior quality of the seeds resulting in the stunted growth of the cultivated cocoa nurseries. Seeds represent the initial growth of plants; therefore, providing seeds with special treatment is crucial for promoting optimal seed growth. The application of Plant Growth Promoting Rhizobacteria (PGPR) microorganisms can stimulate the production of indoleacetic acid (IAA) in plants, leading to enhanced plant growth quality. It can also enhance seed germination (Hardiansyah *et al.*, 2021). The application of rhizobacteria combined with fertilizer has been reported to enhance the growth of cocoa plants (Hastuti *et al.*, 2023). Thus, microorganisms are necessary due to their specific functions to enhance soil fertility.

The study identified 25 distinct bacterial isolates, predominantly gram-negative rods, that may have a significant impact on bio stimulation and biofertilization. Significantly, a number of isolates exhibited characteristics suggestively to be Plant Growth Promoting Rhizobacteria (PGPR), which are recognized for their capacity to improve seed germination, stimulate plant growth, and increase the availability of nutrients in the soil. The production of cocoa in Indonesia is often hindered by soil degradation caused by the decrease in organic matter and the nutrient depletion due to excessive agricultural practices. This study highlights the role of rhizobacteria to enhance soil fertility through the organic waste decomposition, nitrogen fixation, and phosphate solubilization. As a result, it potentially counteracts the negative effects of intensive agriculture on soil health.

Rhizobacteria are non-pathogenic bacteria found in the rhizosphere, which is the area around plant roots. These bacteria have the ability to produce growth hormones, bio stimulants, and fertilize the soil. Additionally, they may break down organic waste, dissolve phosphate, and fix nitrogen. Rhizobacteria engage in significant and extensive interactions with plants, soil, and soil microfauna. It is a valuable source of carbon that may be exploited, and it facilitates multiple nutrient cycles (Hafsah, 2022; Shaikh SS, 2018). Beneficial PGPR, which reside freely in the rhizosphere, exert a direct or indirect favorable impact on plant growth and development. In the cocoa plant, PGPR excretes phytochemical compounds, including hormones, anti-pathogenic chemicals, and diverse beneficial compounds for the plants (Basu *et al*., 2021). Soil microorganisms play a crucial function in providing nutrients to plants and promoting optimal growth and development of cocoa plants based on their specific nutrient requirements and characteristics.

2. Methodology

2.1 Sample Location

The soil samples and cocoa plant diseases in this study were collected from the kaluti cocoa plantation in Meko Village, Pamona Barat District, Poso Regency. The location of the plantation is at 1.8819 degrees South Latitude and 120.4878 degrees East Longitude.

2.2 Soil Preparation

The soil suspension began by preparing a measuring cup and adding 90 ml of distilled water. Afterwards, it took a soil sample weighing 10 grams and placed it in an Erlenmeyer flask containing 90 ml of distilled water. The mixture was vigorously shaken using a Vortex Mixer for one minute. This solution was labeled 10^{-1}. The next phase was transferring 1 milliliter of the suspension into a test tube holding 9 milliliters of distilled water. It was then labeled as 10^{-2}. The dilutions were prepared with a dilution factor of 10^{-6}. The coding system for land location includes cocoa plants adjacent to cover crops (GL), cocoa plants near forests (BL), cocoa plants surrounded by sugar palm trees (JL), and cocoa plants isolated from other plants (KL). Next, the soil suspension was inoculated into a petri dish containing 200 µl of Luria Bertani Agar (LBA) media. Finally, samples were incubated for four days, or until noticeable variations in the appearance of the colonies were observed, indicating clear variances in their morphology. (Sastrahidayat, 2012).

2.3 Medium Preparation

The medium preparation began by weighing the following materials: 10 grams of peptone, 5 grams of yeast extract, 5 grams of NaCl, and 15 grams of bacteriological agar. Subsequently, All the ingredients were combined in a liter of distilled water, followed by applying heat using a magnetic stirrer until complete dissolution. Next, the media was sterilized in the autoclave at a temperature of 121ºC for 15 minutes. It then was poured into the petri dish and cooled it down. The process of pouring the medium was carried out in a controlled environment known as a Laminar Air Flow.

2.4 Bacterial Isolation

The bacterial isolation began by cultivating a certain type of bacteria on fresh media to create the growth of a solitary colony. This selection was based on distinguishing characteristics, such as size, shape, colony surface, and colony margins (Kurahman et al., 2020).

2.5 Bacterial Cell Morphology

Smears of bacteria from pure cultures grown on solid media were prepared. The process of collecting bacteria was carried out by applying a single dose on the glass object, followed by 1-3 drops of distilled water. Afterwards, the sample was flattened and dried. Gram staining was performed after the drying process to color the cells so as to ease the observation under a microscope. The slide was treated with a purple crystal solution for one minute, washed with water, and subsequently exposed to Lugol's solution for one minute. Following the administration of Lugol, the subject was later treated with acetone-alcohol and then exposed to safranin dye for a minute. Finally, the subject was rinsed with water (Goldman & Green, 2009). Once the specimen was subjected to gram staining, it was subsequently observed under a microscope at a magnification range of 400-1000x.

3. Results

3.1 Bacterial colony morphology

A total of 101 bacteria was extracted from cocoa roots and cultured on LB agar media. During the initial phase of isolation, there were a total of 61 bacteria subsequently cultivated in a different environment. Growing bacteria were selected based on the similarity of their morphological characteristics and odor by picking bacteria that do not generate a noxious stench. In the second phase of the screening process, 25 specific bacterial isolates were examined to determine their suitability as a biological control agent. The colonies were acquired and subsequently classified according to their morphology based on the morphological code. The 25 detected isolates showed a wide range of morphological characteristics, such as round and irregular shapes, rough and dull and slimy surfaces, cloudy and colored (yellow and reddish) appearances, and flat, raised, convex, and bumpy elevations. Tabel 1 shows bacterial colony morphology

Tabel 1. Bacterial colony morphology

Isolate Code	Isolate Morphology				
	Shape	**Edges**	**Surface**	**Color**	**Elevation**
GL61	Circular	Entire	Rough/mucoid	Dull	Raised
GL62	Circular	Entire	Rough	Berawan	Raised
GL63	Circular	Lobate	Rough	Dull	Raised
GL64	Circular	Entire	Mucoid	Dull	Raised
GL51	Circular	Entire	Rough	Cloudy	Raised
GL52	Circular	Undulate	Rough	Dull/yellowish	Raised
GL41	Circular	Entire	Rough	Dull/Reddish	Raised
GL41et	Punctiform	Entire	Mucoid	Dull/reddish	Raised
GL42	Circular	Entire	Rough/Smooth	Dull	Raised
GL43	Circular	Entire	Rough	Dull/cloudy	Raised
BL61	Circular	Entire	Rough	Cloudy	Convex
BL62	Spindle	Entire	Rough	Dull/Reddish	Raised
BL63	Circular	Entire	Rough	Dull/white	Flat
BL64	Circular	Entire	Rough	Dull	Raised

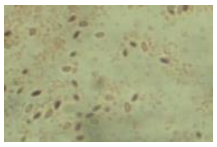
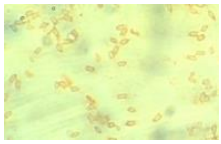
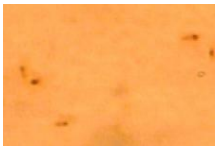
Isolate Code	Isolate Morphology				
	Shape	**Edges**	**Surface**	**Color**	**Elevation**
BL65	Circular	Lobate	Mucoid	Dull	Convex
BL51	Circular	Entire	Mucoid	Dull	Raised
BL52	Circular	Entire	Rough	Dull	Convex
BL41	Circular	Entire	Rough	Dull	Flat
BL42	Circular	Entire	Rough	Dull	Umbonate
JL31	Circular	Entire	Rough	Cloudy	Raised
JL32	Circular	Curled	Rough	Yellowish	Raised
JL21	Circular	Erose	Rough	Dull	Raised
JL22	Circular	Undulate	Rough/Smooth	Dull	Raised
KL31	Circular	Rizoid	Rough/Smooth	Dull	Flat
KL32	Circular	Entire	Rough	Dull/white	Raised

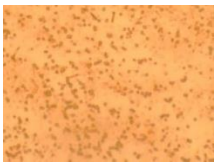
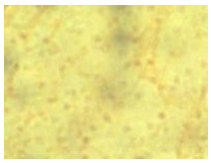
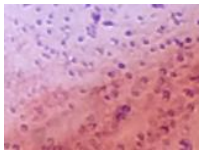
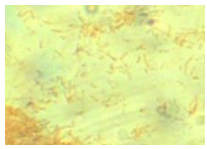
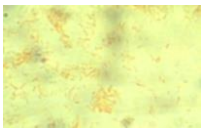
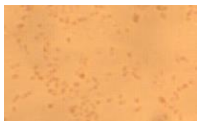
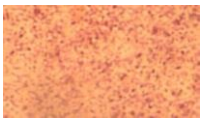
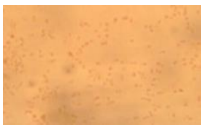
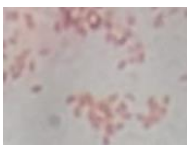
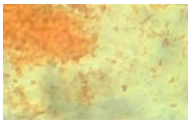
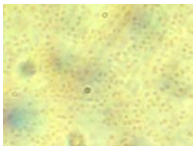
The table above shows that the majority of the colonies demonstrated a circular morphology, with predominantly smooth edges. Additionally, most colonies had a rough surface, dull color, and were situated at a high elevation. Differences in the bacterial colony morphology can be influenced by the growth media, resulting in a similar appearance of bacterial growth upon initial observation.

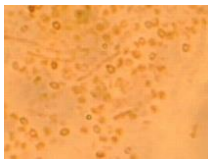
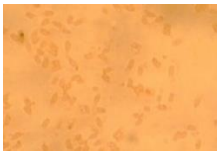
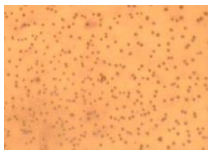
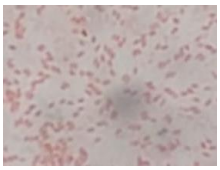
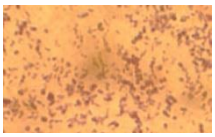
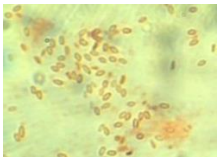
3.2 Gram Stain of Bacteria

Gram stain results showed different types of bacterial cell walls and the shape of the bacterial cell. Tabel 2 shows bacterial morphology and gram stain

Tabel 2. Bacterial morphology and gram stain

Isolate Code	Isolate Morphology		
	Cell Shape	**Gram Stain**	**Picture**
GL61	Bacill	Negative	
GL62	Batang	Negative	
GL63	Batang	Negative	
GL64	Batang	Negative	
GL51	Coccus	Negative	

Isolate Code	Isolate Morphology		
	Cell Shape	Gram Stain	Picture
GL52	Coccus	Negative	
GL41	Coccus	Negative	
GL41et	Too small cell	Negative	
GL42	Bacill	Negative	
GL43	Bacill	Negative	
BL61	Coccus	Negative	
BL62	Bacill	Positive	
BL63	Coccus	Negative	
BL64	Bacill	Negative	
BL65	Bacill	Negative	
BL51	Bacill	Negative	
BL52	Bacill	Negative	

Isolate Code	Isolate Morphology		
	Cell Shape	Gram Stain	Picture
BL41	Bacill	Negative	
BL42	Bacill	Negative	
JL31	Bacill	Negative	
JL32	Coccus	Positive	
JL21	Bacill	Negative	
JL22	Bacill	Negative	
KL31	Bacill	Positif	
KL32	Bacill	Negative	

4. Discussion

Most of the bacteria obtained were rod-shaped, 23 of which were classified as gram negative and three were classified as gram positive. The shape of the cell was primarily determined by the shape of the rod: approximately 19 cells being rod-shaped and the other six being spherical. This diversity of bacteria was essential for their role in the process of nitrogen cycling in cocoa fields and for maintaining an optimal equilibrium of soil organisms. The close interactions between plant hosts and their associated microbes played a crucial role in regulating the plant health. These interactions can enhance the plant's ability to tolerate both biotic and abiotic stresses and diseases (Simmons *et al.*, 2018). Bacterial colonies around plant roots can enhance nutrient availability and confer resistance against diseases. The soil's bacterial variety played a crucial role as each species had its unique function. The diversity of bacteria in a field had an impact on the ability of plants to withstand attacks by harmful microbes that can target cocoa plants. The observed variation in morphology among the isolates indicated a wide range of

functional capabilities, including various stress tolerances and cycle nutrients, which are crucial for maintaining a healthy rhizosphere. This diversity can contribute to cocoa crop productivity by creating a more resilient soil ecosystem that supports plant growth and reduces susceptibility to diseases. The findings of this study are in line aligned with and build upon the previous research conducted by Basu *et al.* (2021), which detailed the role of PGPR in sustainable agriculture. This research suggests a customized method of biofertilization that is seamlessly integrated into existing cocoa farming methods in Indonesia by identifying and separating native rhizobacteria with beneficial characteristics, providing a sustainable substitute for chemical fertilizers and pesticides.

5. Conclusions

A total of 25 bacterial isolates were successfully obtained from the soil surrounding cocoa roots. The detected 25 isolates exhibited diverse morphologies, including round and irregular shapes, rough and dull and slimy surfaces, cloudy and colored (yellow and reddish), and flat, raised, convex, and bumpy elevations. A total of 23 isolates were gram negative while the rest two were gram positive.

Author Contributions

Conceptualization, I. G. P. W and I. K. S.; methodology, I. K. S.; software, P. K. K.; validation, N. S., and I. K. S.; formal analysis, P. K. K.; investigation, P. K. K.; resources, N. S.; data curation, N.S.; writing—original draft preparation, N. S.; writing—review and editing, I. G. P.; visualization, P. K. K.; supervision, I. K. S.; project administration, P. K. K.; funding acquisition, I. G. P. W. All authors have read and agreed to the published version of the manuscript.

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Data Availability

Not applicable

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Conflicts of Interest

Authors The authors declare there no conflict of interest

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