

Research Article

Microbial exploration of the origin of the Pandeglang cocoa plant (*Theobroma cacao* L.) rhizosphere as potential biofertilizer

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Abstract: This study aimed to determine the ability of microorganisms originating from the root ecosystem (Rhizosphere) of cocoa (*Theobroma cacao* L) as a potential superior to biological agents. For the experiment, soil samples were observed from the rhizosphere of cocoa plants in Pandeglang Regency. At this stage, the research was focused on finding out the extent of microbe screening results from cocoa rhizosphere soil in producing phytohormones. The results from the initial selection showed a population of *Azotobacter* 2.8×10^5 cfu/mL, a population of *Azospirillum* of 0.3×10^2 cfu/mL, and a population of Phosphate-solubilizing bacteria (PSB) 4.1×10^7 cfu/mL. In the hemolysis test conducted for 9 isolates, only 1 isolate showed a negative result. From the test of the ability to produce phytohormones showed respectively from the highest values were IAA 5.467 mg/L, gibberellin 3.768 mg/L, zeatin 1.321 mg/L, and kinetin 0.886 mg/L. The land rhizosphere of Pandeglang cocoa plant had superior potential microbes which could be used as biological fertilizers.

Keywords: *Azospirillum*, *phosphate-solubilizing bacteria*, *phytohormone*, *rhizosphere*, *total Azotobacter*

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Introduction

Indonesia is the third cocoa (*Theobroma cacao* L) producer after Ivory Coast and Ghana, however, cocoa that is cultivated by farmers in Indonesia is still facing problems of productivity and product quality (Zahara et al., 2020). One of the problems with the productivity of the cocoa plant is the decline of the quality of health and soil fertility due to the accumulated use of inorganic fertilizers. This problem relates to the product quality where the cocoa plant cannot grow optimally declining the standard of the quantity and quality of plant production. On the other hand, plant productivity decreases mainly due to a lack of soil fertility from

the continuous use of inorganic fertilizers resulting in a decrease in the ecological quality of the land. According to Makarim and Suhartatik (2006), to overcome the decline in land production and productivity, cultivation technology is needed, which in principle can suppress the use of inorganic fertilizers. Therefore, the exertions are needed to improve land conditions biologically by utilizing soil biotechnology (i.e. soil microorganisms and natural fertilizer technology) (Kumalawati et al., 2018). Overall, the habitat for useful microorganisms is found in the soil around plant roots (rhizosphere), which can increase the activity and number of organisms. Several rhizosphere microorganisms play an important role

in the nutrient cycle and soil formation process, plant growth, influence the activity of microorganisms, as well as a biological control against root pathogen (Nildayanti, 2018). Rizobakteri can be found in the plant's rhizosphere environment, where a thin layer of soil envelops the surface of the root and exerts a positive or negative influence on plant growth. (Ernita et al., 2015). The more dense and dense the roots of a plant in the soil, the richer the content of organic matter in the rhizosphere, the denser the population of soil microbes (Tambingsila, 2016).

Exploration of beneficial microorganisms in the rhizosphere of the cocoa plant is expected to be able to find potential isolates as biological fertilizers that produce phytohormones that can improve plant growth. For example, phosphate-solubilizing bacteria (PSB) contained in biological fertilizers have the ability to produce phytohormones such as auxin, gibberellin, and cytokines (Setiawati et al., 2014). According to Firmansyah et al. (2015), bacteria such as *Azospirillum* and *Azotobacter* play an important role in nitrogen fixation in nature. It is known that the main mechanism for improving plant growth by *Azospirillum* is through N fixation. According to Cahyadi and Widodo (2017), biological fertilization is profitable in the long term because the results of their research proved that biological fertilizers could increase soil nutrient content. Following the results of previous research on the potential of plant rhizosphere microorganisms, this study aimed to explore the ability of microorganisms from the rhizosphere of cocoa (*Theobroma cacao* L) as potential superior biological agents. It is known that microbes will be more optimally used if they are suitable for their host plants. Therefore, this study focussed on finding of biological agents that can increase the productivity and quality of cocoa plants. In addition, the use of biological agents from the cocoa rhizosphere will maintain soil health and fertility and balance the microclimate of the cocoa plants. The ability of rhizobacteria to increase the growth and yield of plants depends on the type of rhizobacteria and the type of plant (Adianto, 2017). At this stage, the study was focused on finding out the extent of microbe screening results from cocoa rhizosphere soil in producing phytohormones. For the next stage, another series of studies will be carried out on the application on cocoa seeds.

Materials and Methods

The research was conducted from September 2019 to May 2020. Soil sampling was carried out on cocoa cultivation in Sukajaya Village, Koroncong District, Pandeglang Regency, Banten Province.

Soil samples were taken in a composite then put in airtight plastic and stored in a cool box during the trip to the laboratory. Population count tests for *Azotobacter*, *Azospirillum*, and phosphate-solubilizing bacteria (PSB) were carried out at Laboratory of the Soil and Agro-climate, Faculty of Agriculture, University of Sultan Ageng Tirtayasa, Serang, Banten and Laboratory of Microorganism Conservation, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB-Biogen), Cimanggu, Bogor. Furthermore, a series of analyzes of microbes from the cocoa rhizosphere were carried out to determine the population of *Azotobacter*, *Azospirillum*, and PSB, hemolysis pathogenicity tests, and the ability of microbes to produce phytohormones. A series of analyzes are described in Table 1.

Table 1. A series of analyzes of microbes from the cocoa rhizosphere.

Parameter	Method/ Media	Unit
<i>Azotobacter</i>	LG	CFU/mL
<i>Azospirillum</i>	Nitrogen Free Bromothymol blue	CFU/mL
Phosphate-solubilizing bacteria (PSB)	Pikovkaya	CFU/mL
Hemolysis test	Blood agar	+/-
Pathogenicity test	Tobacco leaves	+/-
Producing phytohormones	Linskens and Jackson (Linskens and Jackson, 2012)	ppm

Population calculation of *Azotobacter*, *Azospirillum*, and phosphate-solubilizing bacteria (PSB)

A soil sample collected from the cocoa plant rhizosphere was weighed as much as 5 grams, and placed in a 100 mL Erlenmeyer flask that has been filled with 45 mL sterile physiological salts. The sample was then homogenized using vortex and let to stand for 10 minutes. Dilution was carried out at 10^{-2} , 10^{-5} , 10^{-7} . One millilitre of each dilution result was then poured in a petri dish and added with specific growing media such as LG for *Azotobacter*, NFB for *Azospirillum*, and Pikovkaya for phosphate-solubilizing bacteria each as much as 15 mL. The petri dish was then shaken until the specific media and dilution results were mixed well. They were then incubated for 2 x 24 hours at 37°C. The number of microbes was calculated using colony counters. Observation of

the populations of *Azotobacter*, *Azospirillum* and PSB were carried out with two replications and noticed the highest value. Three isolates from each selective media were taken from the results of observations of the *Azotobacter*, *Azospirillum*, and PSB populations based on the development of the form and growth of the most dominant isolates so that the isolates were stored as stock on the tilted agar media for a series of further tests. The stock was stored at 4°C and always regrown every three months.

Hemolysis and bacterial pathogenicity test

Hemolysis and pathogenicity tests were carried out to minimize the negative effects of using rhizosphere-based bacteria on plants, animals and humans. Tests were conducted using blood agar medium (from goat) and tobacco leaves. The blood agar is a medium for distinguishing bacteria based on the ability to lyse red blood cells. The hemolysis test was carried out by inoculating a loop of isolate on the surface of the blood agar medium, then incubating it at 37 °C for 18-24 hours. If there are symptoms of hemolysis, it will form an inhibition zone (clear zone) around the growth zone of isolates tested on the media so that the blood. In the pathogenicity test, bacterial isolates were inoculated onto NB media for two days. The bacterial suspension was then injected into the abaxial side of the tobacco leaves, and the effect was observed after 48 hours by looking at necrotic

symptoms in tobacco leaves. A positive control (pathogenic bacteria) was also included to see a clear difference between the pathogenic isolates and not in the final test results.

Growing hormone production test

Measurement of microbe isolates capability to produce phytohormones was made using the modified Linskens and Jackson method. Pure isolate samples that have been grown in NB media were harvested and homogenized with liquid N₂, and 20 mL solvent MeOH 65%, followed by dysentery at 4000 rpm for 30 minutes or filtered using Whatman 42. The supernatant was then taken out and filtered using millipore. Samples of 5-10 µL were then injected into an HPLC.

Results and Discussion

The population of *Azotobacter*, *Azospirillum*, and PSB

The results calculation of functional microbial populations in soil samples of cocoa plant rhizospheres showed the highest population was PSB, respectively followed by *Azotobacter* and *Azospirillum* (Table 2). Data presented in Table 2 show that the soil rhizosphere of cocoa plants has a population of functional bacteria to continue to be studied so that its function can be optimally beneficial for cocoa plants.

Table 2. The results of population counting of *Azotobacter*, *Azospirillum*, and PSB.

Sample	Population of <i>Azotobacter</i>	Population of <i>Azospirillum</i> (cfu/mL)	Population of PSB
Rhizosphere Soil	2.8 x 10 ⁵	0.3 x 10 ²	4.1 x 10 ⁷

According to Manuhuttu et al. (2014), some microorganisms that are commonly used as biological fertilizers include *Azotobacter sp.* and *Azospirillum sp.* as microbes that can fix N from free air. *Bacillus sp.* and *Lactobacillus sp.* can help the fermentation process of organic matter into lactic acid compounds that are absorbed by plants; *Aspergillus sp.* is a phosphate-solubilizing microorganism; *Trichoderma sp.* can be used a fungicide, and *Rhizobium sp.* can form root bindles on plants. Results of this study may be used as one of the references for the next studies in exploring beneficial microbes from cocoa plant rhizosphere, both as nitrogen fixers and phytohormone producers. Some researchers (Simarmata et al., 2004; Maslahat and Suharyanto, 2005) reported that microbes producing growing hormones are very beneficial to enhance plant growth.

Hemolysis and pathogenicity test

The results of testing in the media so that the blood show that one isolate that developed clear zone (inhibition zone) was the LG-2 isolate, then 8 (eight) isolates that passed the hemolysis test were subjected to pathogenicity test. The results showed that there were no symptoms of necrosis on all segments of the leaves applied so that providing a conclusion that the isolate did not give a negative effect on the plant. Results of the hemolysis and pathogenicity tests are presented in Figure 1 and Table 3. Data in Table 3 indicate that the selected microbial isolates from selective media showed dominantly negative results in the hypersensitivity and pathogenicity tests. This illustrates microbes that can grow on selective media in addition to having the potential as expected microbes as the

selective media are also expected not to have a negative impact during application to plant and its environment represented by animals. This is important to know because basically on the

application the microbial isolate suspension is given at high colony density according to the standard of biological fertilizers as $> 10^7$ cfu/mL.



Figure 1. Hemolysis test on the blood agar media and pathogenicity test on the tobacco leaf.

Table 3. The results of hemolysis and pathogenicity test.

No.	Isolate Code	Type of test	
		Hemolysis (Blood agar media)	Pathogenicity (Tobacco leaf)
1	Pikov-1	Negative	Negative
2	Pikov-2	Negative	Negative
3	Pikov-3	Negative	Negative
4	NFB-1	Negative	Negative
5	NFB-2	Negative	Negative
6	NFB-3	Negative	Negative
7	LG-1	Negative	Negative
8	LG-2	Positive	-
9	LG-3	Negative	Negative

Growth hormone production test

Analysis of the ability to produce phytohormones is important to determine the ability of bacteria to produce growth hormones. Growth, development and movement of plants are controlled by several classes of substances which are generally known as plant hormones or phytohormones (Dewi, 2008). Table 4 depicts the complete results of the growth hormone production test from 8 selected isolates. The results showed that all isolates had the

potential to produce the IAA hormone, followed by other hormones. This shows that the isolated bacteria from the rhizosphere of the cocoa plant are very potential as biological agents, both as biological fertilizers and pesticides. The consortium used has a high potency to be applied after a compatibility test has been carried out on the best bacteria according to its characteristics. Biological input in the form of rhizobacteria is needed to avoid a decline in plant health due to chemical input (Hindersah and Simarmata, 2004).

Table 4. Results of growth hormone production test.

No.	Isolate Code	Concentration (mg/L)			
		IAA	Gibberellin	Zeatin	Kinetin
1	Pikov-1	5.131	2.613	1.321	0.543
2	Pikov-2	5.023	3.539	1.198	0.512
3	Pikov-3	4.548	3.399	1.052	0.607
4	NFB-1	5.181	3.437	1.069	0.494
5	NFB-2	5.467	3.056	1.033	0.642
6	NFB-3	3.739	3.798	0.831	0.826
7	LG-1	4.373	3.244	0.551	0.886
8	LG-3	5.412	3.058	0.500	0.560

The use of indigenous microorganism isolates is more recommended in inoculation selection in plants because this microorganism has adapted to local ecological conditions compared to non-indigenous strains (Kizilkaya, 2008). The use of plant growth-promoting rhizobacteria (PGPR) as biological fertilizer is one of the contributions of soil and environmental biotechnology in an effort to increase plant productivity.

One of the PGPR bacteria that is important in the soil ecosystem, especially cocoa plants, is *Azotobacter chroococcum* (Rahmi, 2014). *Azotobacter chroococcum* is a species of rhizobacteria that has been known as an N₂-fixing biological agent, which converts dinitrogen to ammonium through electron reduction and dinitrogen protonation (Kizilkaya, 2008). Other studies have shown that *Azotobacter* is not only effective for nitrogen fixation but can also produce growth hormones, fungicidal ingredients, siderophores, and can dissolve phosphates (Jalilian et al., 2012).

Phosphate solubilizing bacteria have the ability to dissolve inorganic Phosphorus (P) into a form of dissolved phosphate which is available to plants. The dissolving effect is generally caused by the production of organic acids such as acetic acid, formic acid, lactic acid, oxalic acid, malic acid and citric acid produced by microbes. The microbes also produce amino acids, vitamins, and growth-promoting substances such as indole acetic acid (IAA) and gibberellin acid which can increase plant growth (Gyaneshwar et al., 2002). Moreover, P is one of the elements the main plant needed and plays an important role in the process metabolism, so that the presence of PSB is needed by plants.

Various selected microbes from the rhizosphere of the Pandeglang cocoa have the potential to produce phytohormones. This has fulfilled one of the quality standard criteria for biological fertilizers according to the regulation of Ministry of Agriculture Republic of Indonesia no. 01 of 2019. This is a very positive result to be able to continue to trace the ability of microorganisms from the rhizosphere of Pandeglang cocoa as a single isolate or a consortium. The potentials of indigenous microorganisms from Pandeglang can be a reference for making bio-fertilizers and pesticides that can increase the productivity of local farmers' crops.

Conclusion

The rhizosphere of Pandeglang cocoa plant had superior potential microbes which could be used as biological fertilizers or pesticides. The selected microbes showed negative results in the hemolysis and pathogenicity tests, and only one microbe

showed positive results, this showed that the selected microbes were more dominant and did not have a negative impact on animals, humans, as well as plants. The results in the test of the ability to produce growth hormones showed numerical values so that all selected microbes have the potential to produce beneficial phytohormones to support plant productivity.

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