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Characterization of soil microbes in an indicated fusarium wilt-suppressive and -conducive soils in banana plantation at Sukabumi, West Java

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Abstract. Banana plants (*Musa acuminata* L.) on PT Perkebunan Nusantara (PTPN) VIII, Parakansalak, Sukabumi West Java plantation were indicated infected by panama wilt. This disease was caused by *Fusarium oxysporum* f.sp. *cubense* (Foc) resulting the decreases fruit production and quality. However, healthy plants were found on the same areas. This condition may be caused by the differences of soil microbiomes that creates Foc-suppressive or -conducive soils. In this study, bacteria in suppressive (BH) and conducive (BS) soil samples were isolated, and characterized through several tests. The tests consisted of nitrogen fixation, hydrogen cyanide (HCN) production, and Indole Acetic Acid (IAA) production. Sequencing of 16S rRNA gene was done for identification of 26 bacterial isolates. Based on the sequences the bacteria were identified as *Bacillus, Staphylococcus, Enterobacter* and *Lysinibacillus* genus. Several isolates showed their potential as biological agents for Foc, i.e. *Bacillus cereus* (BH 1), *B. cereus* (BH 2), *B. velezensis* (BH 4), *B. cereus* (BH 7), *B. cereus* (BS 12), *B. megaterium* (BS 13), *B.cereus* (BS 15), *B. thuringiensis* (BS 18), *B. vallismortis* (BS 19), *B. bingmayongensis* (BS 20), *B. toyonensis* (BS 22) and *B. pseudomycoides* (BS 25).

1. Introduction

Banana plants are widespread in the Southeast Asia region, including Indonesia. Indonesia has a large area for banana plants, because these plants have many benefits. The benefits of banana plants can be obtained from the fruit, flowers, leaves, stems and tuber [1]. Banana cv Barangan (*Musa acuminata* L.) is one of the most popular banana which planted traditionally and commercially in Indonesia. One of the cv Barangan producers in Indonesia with high yields is PT Perkebunan Nusantara (PTPN) VIII Parakansalak, Sukabumi. However, the production of all types of bananas, including cv Barangan in Parakansalak PTPN VIII tends to be decreasing recently. It is suggested those plants had been infected by pathogenic fungi *Fusarium oxysporum* f.sp. *cubense* (Foc) which causing Fusarium wilt disease. Many banana plants in Parakansalak show wilt symptom and eventually die without producing fruits that are suitable for harvest.

Fusarium wilt disease causes a decrease in the production and quality of fruit from the affected plants. Foc has known as major agent causing decreasing banana production in several are in Indonesia such as in North Sumatra, Riau, Lampung, including banana plantations in PTPN VIII Parakansalak [2]. Disease caused by Foc has succeeded in making the banana cultivar Gros Michel stopped exporting from its home country in 1950 due to nearly extinction [3].The infection of Foc can spread quickly through the

air or land, so if there is no action to handle this situation, all the banana plants left in the Parakansalak are eventually going to die. Furthermore, effective method to control Foc is not available yet.

When we conducted survey at Parakansalak, there were several 'Barangan' plants which were not infected by Foc. This is thought to be caused by differences in soil microbiomes, the major reason causing Foc-suppressive and -conducive soils [4]. Therefore, this study was conducted to determine the characterization of microbial from suppressive (capable of suppressing growth) and conducive soil (supporting growth) to Foc. This study was conducted to obtain information on the potential of microbial isolates to inhibit the growth of Foc which in turn will reduce the infection rate of Foc in banana plantation, especially in PTPN VIII Parakansalak, Sukabumi.

2. Methods

2.1. Soil sampling

Soil sampling was done at PT Perkebunan Nusantara VIII Parakansalak, Sukabumi, West Java. A total of 2 soil samples were taken from 2 different area sites, 1 area indicated Foc-suppresssive soil (BH) and 1 area Foc-conducive soil (BS). As many as 500 g soil of each samples were collected from 3 mixed soil condition located nearby. Soil samples were kept in sterile plastic and kept in cool container before stored in 4 °C in the laboratory. These soils were used as sources of bacterial inoculum.

First of all, 5 g of soil sample was mixed with 45 mL NaCl and homogenized. The solution was then diluted by taking 1 mL of 10^{-1} dilution into a test tube containing 9 mL NaCl to obtain a 10^{-2} dilution, and further dilution was carried out up to 10^{-6}. Each dilution of 10^{-4}, 10^{-5} and 10^{-6} was taken for 1 mL and poured into petri dish containing NA (nutrient agar) medium and then incubated at 32 °C for 24 hr [5].

Bacterial colonies that grow were calculated using Total Plate Count (TPC) based on SNI No. 01-2332.3-2006. Pure cultures were made by taking colonies on the petri dish and inoculated with the quadrant streak method on the new petri dish containing NA media and incubated for 24 hr. Bacteria that grow in quadrant petri dishes will produce single colonies which are then cultured in slant NA media for further characterization test and DNA isolation.

2.2. Characterization of bacterial isolates

2.2.1. Nitrogen-fixing test. The test was done using Jensen media [HiMedia]. Each single isolate was inoculated to Jensen media and incubated for 8 d. Bacteria that grow on the media is considered as nitrogen-fixing bacteria.

2.2.2. IAA-producing bacteria test. Bacterial isolates were cultured in 10 ml sterile Nutrient Broth (NB) + L-tryptophan (200 ppm for 100 mL of NB) medium and incubated in shaker incubator in 100 rpm at 32 ° C for 3 d. Bacterial culture was taken 3 mL and centrifuged at 8000 rpm for 10 min. Supernatant was taken for 2 mL, added with salkowski reagent for 4 mL and 2 drops of orthophosphoric acid, and incubated for 30 min in the dark room. The Optical Density (OD) was measured using a spectrophotometer with a wavelength of 535 nm and IAA concentration was determined based on IAA standard curve [6].

2.2.3. HCN-producing bacteria test. The isolates were cultured on solid King's B media. Each bacterial isolates was isolated and cultured in test tube containing King's B media. Then, the top of the tube is covered by filter paper that has been soaked in solution containing 1% picric acid and 10% Na2CO3 in distilled water. The bacterial culture was incubated for 48 hr. Bacteria that have the ability to produce HCN will make filter paper turned yellow-brownish to red-brown in colour [7].

2.3. DNA amplification by polymerase chain reaction

Bacterial isolates were cultured in 10 mL NB media for 18 hr, then centrifuged at 8000 rpm for 10 min. The pellets were resuspended with 600 µL lysis buffer (containing 60 µL proteinase K (20 mg/mL) in 10 mL lysis buffer) and incubated for 1 hr at 37°C. Phenol: Chloroform: Isoamyl-Alcohol (PCI 25: 24: 1) was then added 600 µL, homogenized and centrifuged at 17 000 g for 5 min. The supernatant was transferred to a new tube and added as much chloroform as the supernatant was taken, then homogenized. After that, centrifugation at 17 000 g for 5 min and the supernatant was transferred to a new tube. Cold isopropanol (3x the volume of the supernatant) was added to supernatant and incubated for 30 min (-20 ºC). After centrifugation at 17 000 g for 15 min the supernatant was removed. The pellet was rinsed using 350 µL of 70% ethanol and centrifuged at 17 000 g for 2 min. The supernatant is discarded and the pellets are dried. DNA was dissolved with 50 µL ddH2O. Isolated DNA was checked by electrophoresis using 1% agarose in TAE buffer with DNA λ as a positive control [8].

Furthermore, bacterial DNA was amplified using polymerase chain reaction (PCR) method using 27F primers (5 '- AGAGTTTGATCMTGGCTCAG - 3') and 1492R (5 '- TACGGYTACCTTGTTACGACTT - 3') [Macrogen]. The reaction was carried out with a final volume of 50 µL consisting of 1 µL of each primer, 25 µL of MyTaq ™ Red Mix, 22 µL free-nuclease water and 2 µL of the DNA template. The reaction was 35 cycles, with 1 min initial denaturation at 95 °C followed by 95 °C denaturation for 15 sec, followed by 57 °C annealing for 15 sec, and 72 °C elongation for 10 sec. The results were visualized by electrophoresis with 1 kb DNA ladder, with an amplification target of 1465 bp.

2.4. Sequence analysis

The product of the PCR was sent to 1st Base, Malaysia to be sequenced with the Sanger dideoxy sequencing method. Sequencing was done using similar primers as for DNA amplification (27F/1492R). The results of sequencing were then analyzed using BLASTN (Basic Local Alignment Search Tool-Nucleotide).

3. Result and discussion

3.1. TPC and soil analysis

Bacterial colonies were found more in samples from healthy soil (BH), *i.e.* 6.75 x 10^5 cfu/g than those from Foc-infected soil (BS), *i.e.* 2.6 x 10^5 cfu/g (Table 1). Although BH samples were more abundance, more isolates were selected from BS samples (Table 1). *Both* soil samples were also measured for its pH, humidity and temperature (Table 2). Soil pH of BH and BS samples were not much different, i.e. 3.3 and 3.5, respectively. Soil pH may influence nutrient solubility. The availability of most nutrients in the soil with acidic pH will decrease, whereas in alkaline pH-soils will increase. It makes BS soil samples have better nutrient availability than BS soil samples [9]. Low pH value in the soil can be caused by microbes or plants respiration that release CO_2 and make the soil more acidic. Rhizosphere, where BH and BS soil samples were collected from, is a soil region with a high microbial content, so the respiration rate is also much higher in this region [10].

The temperature of BH and BS soil samples were 27.8 °C and 24.9 °C, respectively. The optimum growth rate of bacteria and fungi is around the temperature of 25 - 30 °C. At higher temperatures, the ratio of bacteria to fungi will increase [11]. Microbial activity will get higher if the soil temperature is increasing. It makes the temperature in BH samples is better than BS samples in terms of microbial activity rate, because it has a higher value and is in the optimum temperature range for bacterial and fungal growth [12].

Soil humidity with values > 60% is considered to be very suitable for plant growth [13]. The humidity of BH and BS soil samples were 75.80% and 74.60%, respectively; this indicated that both soils were suitable for banana planting. Humidity also affects microorganisms, low humidity results in decreased microbial activity due to stress by dehydration. Low humidity also limits the mobility and supply of

substrate to soil microbes by diffusion through soil solutions. Therefore, BH soil was better because it has higher humidity value than BS soil [12].

Table 1. Total Plate Count (TPC) of soil samples.

No	Soil Sample	TPC (cfu/g)	Selected Isolates
1	BH	6.75×10^5	10
2	BS	2.6×10^5	16

Table 2. Measurement of pH, humidity and temperature of soil samples.

Soil Sample	pH	Humidity	Temperature
BH	3.3	75.80 %	27.8 °C
BS	3.5	74.60 %	24.9 °C

3.2. Characters of bacterial isolates

There were 8 and 10 bacterial isolates from BH and BS soil samples, respectively which showed positive results on the nitrogen-fixing test (Fig. 1). This test was conducted to determine the potential of soil bacteria in fixing nitrogen. Nitrogen is used for plant growth as a component of nucleic acids and proteins. The abundance of nitrogen in the soil needs to be changed first in the form of ammonium (NH^{4+}) and nitrate ions (NO^{3-}) to be used by plants. Therefore, the presence of bacteria in the soil which has the ability to fix nitrogen is very important to support the survival of plants. BH soil sample is better because the ratio of bacteria that can fix nitrogen is greater than in BS samples [14].

The HCN producing test aims to determine which bacterial isolates that can produce HCN. The results showed that there were 6 out of 10 BH isolates and 6 out of 16 BS isolates that positively produced HCN, marked by discoloration of filter paper from yellow to brownish orange. HCN is a substance that associated with biocontrol activities. HCN contributes to resisting pathogen activity, where HCN is toxic to some pathogenic microorganisms [15]. The higher ratio of positive to negative HCN-producing bacteria of BH samples than those of BS samples supports the fact that healthy bananas grew in BH soil.

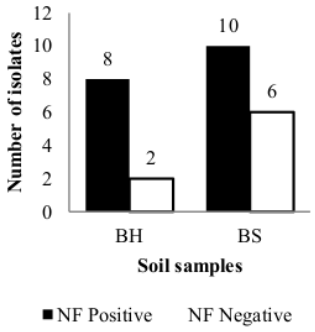

Figure 1. Nitrogen fixation (NF) reaction of bacterial isolates from Foc-infected soils (BS) and healthy soils (BH).

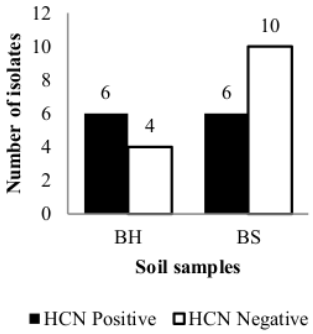

Figure 2. HCN production reaction of bacterial isolates from Foc-infected soils (BS) and healthy soils (BH).

The results of IAA test showed that all isolates could produce IAA, indicated by colour change of the samples from clear yellow to reddish. The bacterial isolate that produced the highest concentration of IAA was BS 23 (98.39 ppm), while the lowest concentration was BH 8 (15.06 ppm). The presence of IAA-producing bacteria can make plant growth better, because the IAA will be absorbed by plants through the roots. Bacteria that produce IAA have benefits for plants, where bacterial inoculation that produces IAA in plants can induce lateral root proliferation and root hair as reported by Mohite [16]. Apart from having benefits in increasing plant growth, it does not mean that IAA-producing bacteria are always profitable. IAA-producing bacteria can also be a pathogenic bacteria [17].

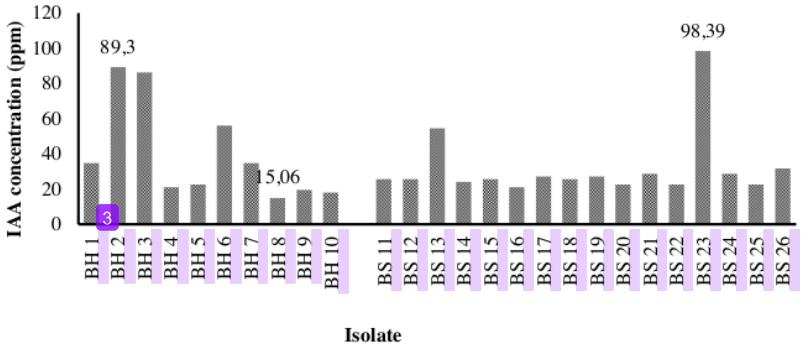

Figure 3. IAA-production test of of 26 total bacterial isolates from Foc-infected soils (BS) and healthy soils (BH).

3.3. Molecular Identification

The bacterial isolates from BH and BS soil samples were identified based on 16S rRNA sequences. The 16S rRNA gene is a conserved gene, which has a length of ~ 1500 bp with 10 conserved regions and 9 hypervariable regions. The area of conserved genes commonly found in all bacteria (conserved region) and only undergoes very small changes over time, while the hypervariable part with different sequences of each type of bacteria which facilitates the identification of species and genus of a bacterium [18].

The search results for bacteria using BLAST-N showed the identity value of bacterial isolates with the smallest and highest value of 96.52% (BS 20) and 100% (BH 1, BH 7 and BS 12), respectively. Bacterial isolates were identified as *Bacillus*, *Staphylococcus*, *Lysinibacillus*, and *Enterobacter* genus. The results of identification of bacterial isolates were dominated by the *Bacillus* genus for bacterial isolates from BH and BS soil samples.

3.4. Bio-control potential of bacterial isolates

Study of microbial diversity and its potential as bio-control agents has been reported in several publications [19]. It was reported previously, that some identified bacteria in this study has been used as biocontrol agent for Foc, namely *B. cereus* (BH 1), *B. cereus* (BH 2), *B. velezensis* (BH 4), *B. cereus* (BH 7), *B. cereus* (BS 12), *B. megaterium* (BS 13), *B. cereus* (BS 15), *B. thuringiensis* (BS 18), *B. vallismortis* (BS 19), *B. bingmayongensis* (BS 20), *B. toyonensis* (BS 220, and *B. pseudomycoides* (BS 25).

Various types of microbes which presence in the soil may interact one to each other. These microbial interaction is influenced by secondary metabolites produced by each microbe, which may have defence, competition, or signal function. Reaction between each secondary metabolites can cause mutualism, competition, antagonistic, pathogenic, or parasitic relationship. It has been reported that microorganisms from the same ecosystem can trigger and induce activation of the biosynthetic path that is previously inactive (silent), resulting in new natural products, and vice versa. This causes the potential of individual

bacteria as biocontrol agents to be lost or inactive due to interactions with other types of microbes in their community structure [29].

Table 3. The potential of *Bacillus* as biocontrol agent for several plant pathogens.

Soil Sample	Species	Bacterial Potential
BH	*B. cereus* BH 1	Has the ability to increase the growth of chili plants and inhibit disease infections by Fusarium and Ralstonia attacks [20]
	B. cereus BH 2	Control *Ralstonia syzigii* subsp. Indonesiensis and supports the growth of chili tomato plants [21]
	B. velezensis strain BH 4	Produces volatile organic compounds that inhibit R. solanacearum and Bacillomycin D which inhibit attacks *F. graminearum* [23]
	B. cereus BH 7	Increase growth and control the infection of R. solanacearum on tomato plants inoculated with this strain bacteria [22], also able to produce IAA and can increase the growth parameters of dry weight in Safflower plants inoculated with this bacterium [24]
BS	*B. cereus* BS 12	Increase tomato plant growth and control fungal infections *R. solanacearum* inoculated on tomato plants [24]
	B. megaterium BS 13	Produces emimycin and oxetanocin antibiotics that can function as antibacterial agents. Its nature as a fungicide can reduce the infection of *Rhizoctonia solani* [25]
	B. cereus BS 15	Improve tomato plant growth and control fungal infections of R. solanacearum inoculated on tomato plants, and control *Ralstonia syzigii* subsp. Indonesiensis and supports the growth of chili and tomato plants [22]
	B. thuringiensis BS 18	Has the ability to control diseases of Ralstonia and Fusarium against chili pepper, supports the growth of chili pepper plants and suppresses wilt caused by Fusarium and Ralstonia [20]
	B. vallismortis BS 19	Produces Bacillomycin D which is antimicroabial [26]
	B. bingmayongensis BS 20	Have the ability to reduce the level of damage to chili plants infected with Fusarium [20]
	B. toyonensis BS 22	Ability to control *Ralstonia syzigii* subsp. Indonesiensis [21]
	B. pseudomycoides BS 25	Supports growth of tomato plants and controls infection by *R. solanacearum* [27]

4. Conclusion

Healthy soils (BH) harboured more bacteria than Foc-infected soil (BS). The genus Bacillus dominated bacterial isolates from both types of soil, i.e. *B. cereus* (BH 1, BH 2, BH 7, BS 12, BS 15), *B. velezensis* (BH 4), *B. megaterium* (BS 13), *B. thuringiensis* (BS 18), *B. vallismortis* (BS 19), *B. bingmayongensis* (BS 20), *B. toyonensis* (BS 22) and *B. pseudomycoides* (BS 25). These isolates have the potential as biocontrol agent for Foc, although further research on interactions between bacterial isolates in a bacterial consortium need to be tested.

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