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Novel microbial consortium formulation as plant growth promoting bacteria (PGPB) agent

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Abstract. PGPB enhance plant growth through nitrogen fixation, IAA production, phosphorus and potassium solubilization. PGPB as biofertilizer provide an alternative for chemical fertilizer to reduce environmental damage. PGPB in consortium could be more effective instead of single strain inoculant. The aim of this study is to investigate compatibility, design soil bacteria consortium isolated from rubber-Canna intercropping plantation and evaluate the consortium's potential as biofertilizer. Functional roles of PGPB was tested such as nitrogen fixation, potassium and phosphate solubilization, and IAA production. Detection of pathogenic bacteria was tested by Blood Agar method. Compatibility test was performed by cross streak method. Bacteria were identified using sequencing of 16srRNA gene and verified using BLAST to database of NCBI. The results showed that among 19 bacterial isolates, all showed the nitrogen-fixing activity, G5 had the highest phosphorus and potassium solubilization index, whereas *Citrobacter braakii* strain 167 produced the highest IAA concentration. Compatibility analysis showed that *Citrobacter freundii* strain LMG 3246, *Citrobacter braakii* strain DSM 17596 and G5 are compatible as a bacteria consortium and may developed as biofertilizer. This study found a possible new and beneficial biofertilizer formulation to enhance plant growth and to reduce the application of chemical fertilizer.

1. Introduction

Intercropping is one of the potential agricultural technologies that can support sustainable farming systems. Intercropping provides various benefits including optimizing the utilization of nutrients, mutual beneficial interactions between plants, inhibiting weeds growth and pest control [1]. Intercropping also provide an increase content of organic matter in the soil. Organic matter can increase the population of microorganisms both in type and amount so that soil quality also increases [2]. The organic carbon content that characterizes the amount of soil organic matter and the total number of bacteria in rubber land planted with canna is higher than that of land without canna. This indicates that planting canna intercrops can affect the total number of bacteria in the soil [3].

Plant Growth Promoting Bacteria (PGPB) is a bacteria that lives free in the soil, it can also be found in the rhizosphere, rizoplane and filosphere [4]. PGPB has several capabilities including nitrogen fixation, dissolving phosphate and potassium elements to increase the availability of nutrients for plants. The capabilities of the PGPB to increase plant growth can make PGPB used as a substitute for chemical fertilizers, which are biofertilizers in agriculture [5]. Chemical fertilizers can make soil structures damaged and reduce levels of soil organic matter [6]. Biofertilizers can help provide certain nutrients for plants, decomposition of organic matter and provide a better rhizosphere environment so



that biological fertilizers are environmentally friendly alternatives that can increase crop productivity [7]. Bacterial isolates as biological fertilizer agents are more effectively used in consortium form than in the single form [8]. Microorganisms used as inoculants of biological fertilizers are nitrogen fixer bacteria, phosphate and potassium solver. These microbes must be tested first to prove that they are not pathogenic so that when we applied it, they are not harmful to the surrounding environment. In this study, we conducted to evaluate the ability of soil bacteria to be used as a consortium as biological fertilizer. Bacteria samples were collected from Rubber-Canna intercropping plantation system with (G) or without *C. Indica* (TG) on the plantation.

2. Materials and methods

2.1. Pathogenicity test

Pathogenicity test of all bacteria were inoculated in Blood Agar Base (HIMEDIA) containing 5% v / v sterile defibrination sheep blood. Each isolate was streaked quadrant and incubated for 96 hours. Observed with light source. The pathogenicity were examined for Beta-hemolysis (clear zones), alpha-hemolysis (green zones) and gamma-hemolysis (no clear zones around colonies) [9].

2.2. Characterization of bacterial isolates

2.2.1 Phosphate-solubilizing bacteria test

Bacteria were inoculated in pikovskaya agar media with addition of tricalcium phosphate incubated for 2-10 days. The clear zones formed by the bacteria on the pikovskaya medium were calculated (solubilization index) using the following equation ($\text{halozone} + \text{colony diameter} / \text{colony diameter}$) [10]

2.2.2. *Potassium-solubilizing bacteria test.* Bacteria were inoculated in Aleksandrov agar media by the spread plate method. The solubilization index calculated by dividing the diameter of the clear zone + colony diameter by the diameter of the colony. Microbial growth was observed for 4 days [11]

2.2.3. *Nitrogen-fixing bacteria test.* Bacteria inoculated into a petri dish containing Jensen's media, then incubated at room temperature for 8 days. Isolates that have the ability to fix nitrogen will grow in Jensen's medium [12]

2.2.4. *IAA-producing bacteria test.* The ability of IAA-producing bacteria was tested by bacteria were cultured in 10 mL of NB media + L-tryptophan (200 ppm for 100 ml NB), incubated for 3 days at 28 ° C. Bacterial culture was taken 3 ml and centrifuged at 8000 rpm for 10 min, supernatant was taken and 4 mL of salkowski reagent was added, incubated in the dark room for 30 minutes. IAA concentration ($\mu\text{g} / \text{mL}$) was determined based on IAA standard curve, using a spectrophotometer with a wavelength of 535 nm to measure optical density (OD) [13]

2.3. Compatibility test

Each isolate was streaked vertically and horizontally on LB agar media. Incubated for 48 hours and observed whether or not there was a lysis between the two isolates. Isolates are compatible if there is no lysis or inhibition zone, isolates are not compatible if there is a lysis or inhibition zone [14]

3. Results and discussion

3.1. Pathogenicity test

The use of bacteria as biological fertilizer agents must fulfill several criteria, one of that criteria is its pathogenicity. Pathogenicity test of bacterial isolates was carried out through hemolysis tests by Blood Agar Media. The results showed that the microbes gave different responses to the pathogenicity. The results of the pathogenicity test showed that 2 isolates that beta hemolysis, 10 alpha isolates and 7 gamma isolates (Table 1). Pathogenic bacteria isolates such as *Bacillus panaciterrae*

strain Gsoil 1517 and isolate G8 are not used for biological fertilizer formulations because it's feared it will be dangerous for plant, animal and human.

Table 1. Hemolysis test of 19 total isolates

Isolates code	Type of Hemolysis
<i>Citrobacter freundii</i> strain ATCC 8090 (TG14)	α
TG15	α
<i>Pseudomonas stutzeri</i> strain VKM (TG16)	γ
<i>Pseudomonas stutzeri</i> strain ATCC 17588 (TG17)	γ
<i>Citrobacter freundii</i> strain JCM 1657 (TG14)	α
TG42	γ
TG43	α
<i>Bacillus panaciterrae</i> strain Gsoil 1517 (TG51)	β
<i>Citrobacter braakii</i> strain 167 (TG61)	γ
<i>Citrobacter braakii</i> strain DSM 17596 (TG62)	α
<i>Citrobacter freundii</i> strain NBRC 12681 (G1)	α
G2	γ
G3	α
<i>Bacillus subtilis</i> strain BCRC 10255 (G4)	α
G5	γ
G6	α
G7	γ
G8	β
<i>Citrobacter freundii</i> strain LMG 3246 (G12)	α

3.2. Characters of bacterial isolates

There were 8 and 3 from G and TG soil samples, that were showed positive results to dissolve phosphate (Fig 2). This test was conducted to determine the potential of soil bacteria in solubilizing phosphate. There have been several reports of *Pseudomonas*, *Bacillus* and *Citrobacter* species with their ability to solubilize phosphate. Yadav *et al.*, [15] found that *Pseudomonas* categorized as phosphate solubilizer bacteria [15], Rathore *et al.*, [16] reported *Citrobacter* and *Bacillus* have an ability to solubilize phosphate tested with pikovskaya medium with addition of tricalcium phosphate as a P source. The ability of *Bacillus subtilis* also reported [17][18] Each isolate has a wide variety of solubilization index. G5 had the highest phosphorus solubilization index (2.38 ± 0.57), while the lowest G8 (1.12 ± 0.07).

The clear zone area qualitatively can show the size of the ability of the ability of BPF to dissolve P from insoluble phosphate [18]. Phosphate solvent bacteria have the ability to increase the mobility or solubility of inorganic P compounds, so that P is transformed into an available form and can be absorbed by plants [19]. Phosphate microbial inoculants can contribute about 20-25% of the phosphate requirement for plants. Phosphorus is important for flower or fruit formation in plant growth [20]

The Potassium-solubilizing test was conducted to determine which bacterial isolates can solubilize potassium. The results showed that 2 and 5 from G and TG soils showed positive results. There have been several reports of *Pseudomonas*, *Bacillus* and *Citrobacter* species with their ability to solubilize potassium. Prajapati & Modi reported that Family Enterobacteriaceae have the ability to solubilize potassium *Bacillus* [21] and *Pseudomonas* also reported as potassium solubilizer bacteria [22][23]. Each isolate has a wide variety of solubilization index, isolate G5 had the highest phosphorus solubilization index (2.38 ± 0.57), while the lowest *Citrobacter freundii* strain ATCC 8090 (0.78 ± 0).

BPK will dissolve potassium bound by silicate (Si) into a form available to plants. Potassium is one of the three essential plants macronutrients along with nitrogen and phosphate. Potassium enhance the ability of formation and transport carbohydrates, plant to resist pests and disease, strengthen the stem, helps the development of healthy plant roots, photosynthesis, transportation of assimilation results, enzymes and minerals [24].

The results of nitrogen-fixing test showed that all isolates could fixation nitrogen. Nitrogen- fixing

test was conducted to determine the potential of bacteria in fixing nitrogen. Nitrogen is a nutrient essential for plants used for the formation of chlorophyll, protoplasm, protein, and nucleic acids [25]. Nitrogen-fixing bacteria enhance plant growth with increase or improve the content of nitrogen in the soil and produce growth promoting substances [26].

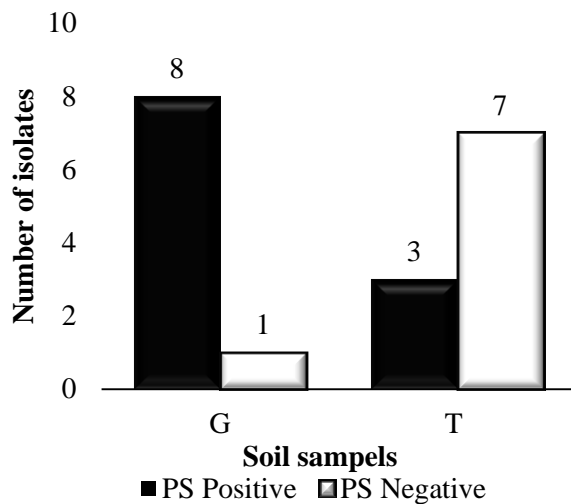


Figure 1. Phosphate Solubization (PS) Results of bacterial isolates from Ganyong soils (G) and Without Ganyong soils (TG)

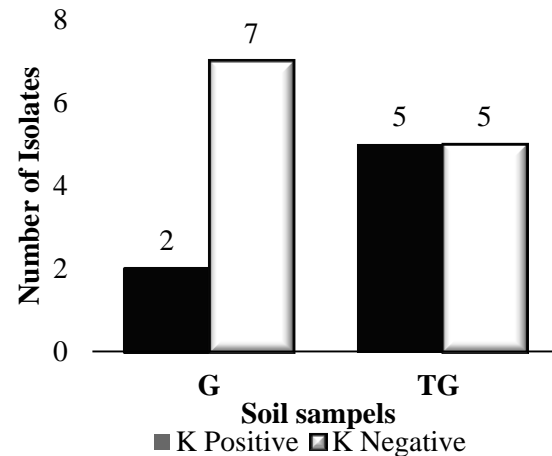


Figure 2. Potassium Solubization (K) Results of bacterial isolates from Ganyong soils (G) and Without Ganyong soils (TG)

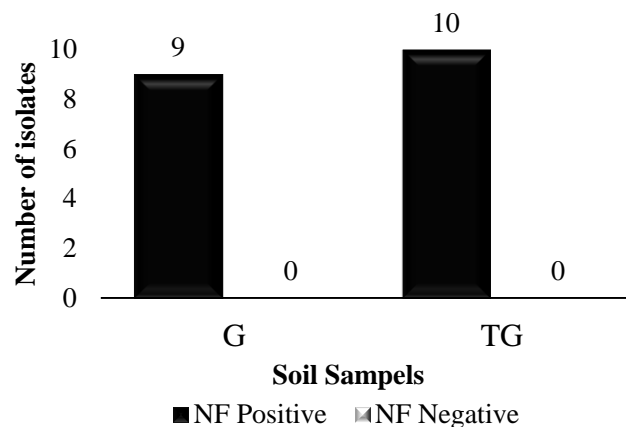


Figure 3. Nitrogen Fixation (NF) Results of bacterial isolates from Ganyong soils (G) and Without Ganyong soils (TG)

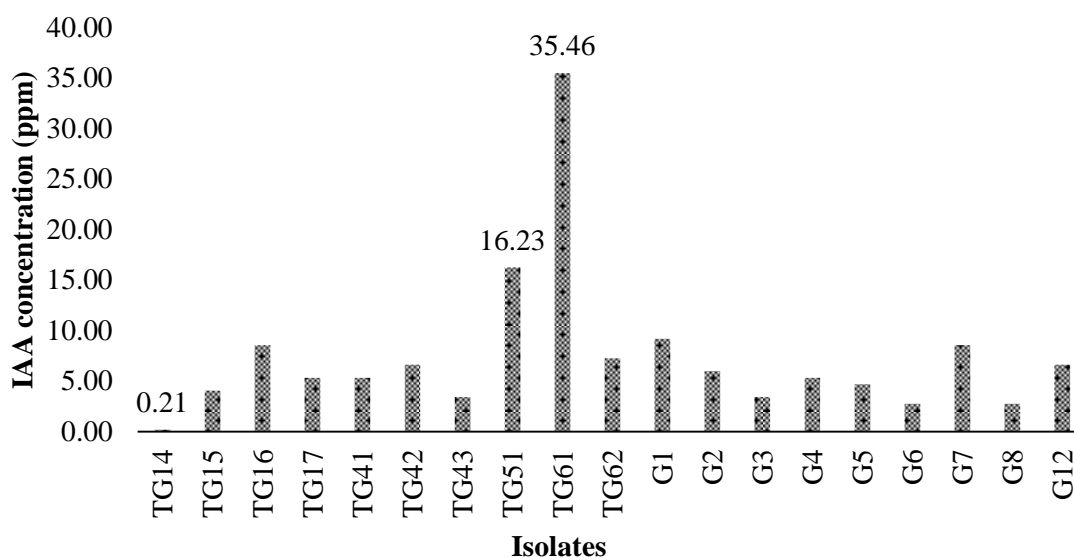
The results of IAA test showed that 7 and 6 isolates from G and TG could produce IAA, indicated by color change of the samples from clear yellow to reddish. The bacterial isolate that produced the highest concentration of IAA was *Citrobacter braakii* strain 167 (TG61) (35.461 ppm), while the lowest concentration was *Citrobacter freundii* strain ATCC 8090 TG14 (0.205 ppm). IAA-producing bacteria directly can increase the rate of plant growth, IAA produced by bacteria also plays a role in stimulate initiation of roots [27].

Table 2. Qualitative estimation of phosphate solubilization

Isolates	Solubilization index (SI)
<i>Pseudomonas stutzeri</i> strain ATCC 17588 (TG17)	1.12 ± 0.09
<i>Bacillus panaciterrae</i> strain Gsoil 1517 (TG51)	1.46 ± 0.32
<i>Citrobacter freundii</i> strain NBRC 12681 (G1)	1.12 ± 0.09
G3	1.96 ± 0.31
<i>Bacillus subtilis</i> strain BCRC 10255 (G4)	1.18 ± 0.16
G5	2.38 ± 0.57
G6	1.34 ± 0.25
G7	1.34 ± 0.19
G8	1.12 ± 0.07
<i>Citrobacter freundii</i> strain LMG 3246 (G12)	1.34 ± 0.11

Table 3 Qualitative estimation of potassium solubilization

Isolates	Solubilization Index (SI)
<i>Citrobacter freundii</i> strain ATCC 8090 (TG14)	0.78 ± 0
<i>Pseudomonas stutzeri</i> strain VKM (TG16)	1.06 ± 0.02
TG42	1.12 ± 0.09
<i>Bacillus panaciterrae</i> strain Gsoil 1517 (TG51)	1.13 ± 0.04
<i>Citrobacter braakii</i> strain DSM 17596 (TG62)	1.07 ± 0.03
G5	1.28 ± 0.07
<i>Citrobacter freundii</i> strain LMG 3246 (G12)	1.17 ± 0.05

**Figure 4.** IAA-production test of 19 bacterial isolates from Ganyong (G) and without Ganyong (TG) soil.

3.3. Compatibility test results

Based on characters of bacterial isolates, four consortium design combinations were obtained, coded K1, K2, K3, K4. The design of the consortium is carried out compatibility tests to determine the synergy between bacteria. The results of bacterial compatibility tests can be seen in Table 4.

Table 4. Compatibility test result of selected bacteria from pathogenicity test and characterization of bacterial isolates

Combination 1			
	<i>Citrobacter freundii</i> strain LMG 324 (G12)	G%	<i>Citrobacter braakii</i> strain DSM 17596 (TG62)
<i>Citrobacter freundii</i> strain LMG 324 (G12)		+	+
G%	+		+
<i>Citrobacter braakii</i> strain DSM 17596 (TG62)	+	+	
Combination 2			
	<i>Citrobacter freundii</i> strain NBRC (12681) (G1)	G3	G7
<i>Citrobacter freundii</i> strain NBRC (12681) (G1)		+	+
G3	+		+
G7	+	+	
Combination 3			
	<i>Citrobacter freundii</i> strain ATCC (8090) (TG14)	TG42	<i>Pseudomonas stutzeri</i> strain VKM (TG16)
<i>Citrobacter freundii</i> strain ATCC (8090) (TG14)		+	+
TG42	+		+
<i>Pseudomonas stutzeri</i> strain VKM (TG16)	+	+	
Combination 4			
	<i>Citrobacter freundii</i> strain JCM 1657	<i>Citrobacter braakii</i> strain 167 (TG61)	
<i>Citrobacter freundii</i> strain JCM 1657		+	
<i>Citrobacter braakii</i> strain 167 (TG61)	+		

The selected bacteria from pathogenicity and several potential bacteria screening were tested its compatibility with cross streak method. No lysis was found at the juncture from all combinations. It is reported that all isolates compatible with each other. All combinations showed compatibility with each other because the bacteria is able to provide nutritional metabolites, that nutrients can be used by other bacteria to grow so bacteria. The main factor that bacteria need to be able to work well together is synergism [28]. Compatibility or synergism is an important thing because the antagonistic relationship between microorganisms can lead to instability in the consortium and cannot obtain the expected function of the consortium [29]. The combination of bacteria (consortium) can produce more benefits and work more effectively to increase plant growth compared to single or single strain bacteria [32]. Biofertilizer usually applied in the form of a consortium, each consortium can play a role to enhance plant growth.

4. Conclusion

Based on this study we conclude that, from the functional test among 19 bacterial isolates, all showed the nitrogen-fixing activity, G5 had the highest phosphorus and potassium solubilization index, whereas *Citrobacter braakii* strain 167 produced the highest IAA concentration. Compatibility analysis showed that *Citrobacter freundii* strain LMG 3246, *Citrobacter braakii* strain DSM 17596

and G5 are compatible as a bacteria consortium and may developed as biofertilizer because their ability of fixation nitrogen, solubize phosphate and potassium. This study found a possible new and beneficial biofertilizer formulation to enhance plant growth and to reduce the application of chemical fertilizer.

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