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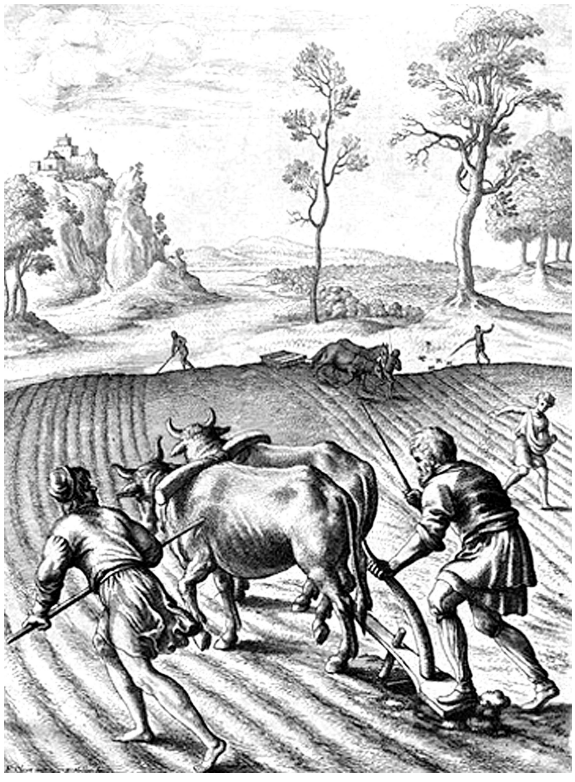

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Potency of Plant Growth Promoting Endophytic Bacteria from Rubber Plants (*Hevea brasiliensis* Müll. Arg.)

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Abstract: Endophytic bacteria is bacteria living in plant tissue and can be isolated through sterilization of tissue surface. Exploration of endophytic bacteria from rubber plant, that are potentially involved in enhanching growth, is important to be carried out. The objective of this experiment was to characterize and select the endophytic bacteria from rubber plants that had potency to enhance growth. Endophytic bacteria were isolated from sample taken from leaf, shaved bark and feeder root of IRR 118 and IRR 39 rubber clones. The 117 endophytic bacteria were found from isolation and then they were selected by hypersensitive response and hemolysis test and through germinating and growth test, the best five endophytic bacteria were selected. Nitrogen fixation of endophytic bacteria indicated by ARA method showed that acetylene reduction ranged from 28.43-42.30 nmol μL^{-1} h^{-1}. The capacity to produce Indole Acetic Acid (IAA) was 28.167-119 μg mL^{-1}, gibberellin 7.5-60 μg mL^{-1}, cytokinin (zeatin) 0.012-0.025 μg mL^{-1} and cytokinin (kinetin) 0.004-0.029 μg mL^{-1}. Identification of five bacteria based on partial sequencing 16S rRNA had found *Bacillus cereus, Pseudomonas aureginosa, Brachybacterium paraconglomeratum, Bacterium* and *Providencia vermicola.* Endophytic bacteria were able to enter to planlet originated from micro cutting proven by Scanning Electron Microscopy (SEM).

Key words: Endophytic bacteria, sterilization of tissue surface, rubber plant, scanning electron microscopy

INTRODUCTION

Endophytic bacteria is organism that lives in association with plant for whole or partial of their life cycle. Endophytic bacteria is living by colonization in inner tissue of plant without causing interference to plant and most of endophytic bacteria are beneficial because they are able to act as biocontrol, make nutrient more available, produce hormone promoting growth and natural product resources for medicine, agriculture and industry (Bacon and Hinton, 2006; Strobel and Daisy, 2003).

Endophytic bacteria as Plant Growth Promoting Bacteria (PGPB) can promote growth by their effect on plant metabolism by providing substances needed by plants. This bacteria can cause N_2 fixation, increase phosphate solubility and iron availability, produce hormone such as auxins, gibberellins, cytokinin, ethylene and absisic acid. Besides that, this bacteria can improve plant tolerance to stresses such as drought, high salinity, metal toxicity and pesticide effect. The role of this bacteria is contributing in better growth and development compared with control without the presence of this bacteria. However, this bacteria can not increase the genetic capacity of plant because genetic subtances are not transferred. Several endophytic bacteria can improve forest tree species such as pine and desert tree like cactus. Endophytic bacteria such as *Pseudomonas fluorescens* and *Bacillus* can act as PGPB-biocontrol to control *Fusarium* pathogen in soil for cotton, *Rhizoctonia solani* and *Sclerotium* for other crops (Bashan and de-Bashan, 2005).

Rubber plant has been grown widely which may indicate its ability to adapt to variable environments and clone variation that may carry potential endophytic bacteria that support plant growth. Exploration of endophytic bacteria that promote growth from rubber tree is important to be carried out. The objective of study was to characterize and select endophytic bacteria that promote growth from rubber tree.

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MATERIALS AND METHODS

Endophytic bacteria isolation: Tissue sample was taken from leaf, shaved bark, feeder roots of IRR 118 and IRR 39 heve a clones planted in 2002 at experimental garden of Sembawa Research Centre, Banyuasin, South Sumatra.

Endophytic bacteria was isolated by using method developed by Hallmann *et al.* (1997) and modified method by Munif (2001). Samples taken from leaf, shaved bark and feeder root were washed by flowing water until they were clean, then they were dried with tissue paper and weighed as much as 1 g. The surface of sample was sterilized by flushing with sterile water for 2 times, then sample was soaked in 70% alcohol for 30 min then leaf sample was soaked in 3% NaOCl for 2 min, while shaved bark and root samples were soaked for 3 min. All samples were then flushed 3 times with sterile water. The sucessfullness of sample surface sterilization could be known by passing samples on the top of Tryptic Soya Agar (10%) and Nutrient Agar (10%) media and petri discs were then incubated in room temperature for 3 days. If there was no microbe growing in surrounding passing area, it indicated that the sample was sterile and sterilization process was sucessfull. Sample was finely ground with sterile mortar and then subsequently diluted with 9 mL sterile physiological water (0.85% NaCl) until reach dilution of 10^{-4}. In each diluted sample, it was taken 1 mL of extract and grown in TSA (10%) and NA (10%) media with 3 replications each media, then they were incubated for 3 days at room temperature and then selected bacteria colony was purified on TSA and NA media.

Hypersensitive Response (HR) test: Hypersensitive response test was carried out by using tobacco as indicator whether endophytic bacteria had potential as pathogen for plant. Endophytic bacteria with density of 10^9 CFU mL^{-1} in liquid media was injected by using 1 mL syringe (without needle) to 3 month old tobacco leaf (*Nicotiana tabacum* L.) and the observation was made until 48 h (Schaad *et al.*, 2001).

Hemolysis test: Hemolysis test was carried out to determine whether endopytic bacteria have potential as a pathogen for human and animal. Endophytic bacteria was grown in blood agar media which had been mixed with sheep blood with 5% concentration from total blood agar media. The test was using positive pathogen bacteria on human or animal and then it incubated for 18-24 h on room temperature (Zimbro and Power, 2009).

Germinating and growth test: Rice seed of Ciherang cultivar was used to test the effect of endophytic bacteria on germination rate than growth. Rubber seed was not used because it was naturally losing the viability very rapidly. Germinating test was done by putting seed on paper then put on petri disc (ISTA, 2010) and the determination of germinating rate was used by method of IRRI (2010). The test method was done by soaking rice seeds in suspension of endophytic bacteria isolate with Optical Density (OD) = 0.1 for 24 h while for control, seeds were soaked in sterile water. Twenty five seeds were put in each petri disc layered with wet filter paper and the test was carried out in 4 replications. Germinating rate was determined after 2 days of incubation, while growth was determined after 5 incubation days. Growth parameters were observed on root and shoot length (Rustam, 2012). Then, five bacteria isolate, that had the best germinating rate and growth, were selected based on scoring.

Test of N_2 fixation: The test of N_2 fixation capacity of endophytic bacteria was done by measurement of N_2 fixation activity which was inderectly measured with Acetylene Reduction Assay (ARA) by using Gas Chromatography (GC). The fixing N_2 bacteria were able to reduce acetylene into ethylene and this change was measured with GC (Gothwal *et al.*, 2007).

Test capacity to produce Indole Acetic Acid (IAA): Endophytic bacteria were innoculated in Luria Berani media and put in shaker with 200 rpm for 5 days at room temperature. Bacteria biomass were separated with centrifuge with 9000 rpm for 15 min. Supernatan obtained was mixed with Salkowski reagent and then it was let to settle for 1 h. The next step was measuring the absorbance with spectrophotometer at wave length of 520 nm (Akbari *et al.*, 2007).

Test of capacity to produce gibberellin: Endophytic bacteria were innoculated on Jensen's Broth media and shaked for 5 days by using a shaker, then they were centrifuged, supernatan was filtered with separating funnel by adding water and pH solution 1-2 (0.1 M HCl), ethyl acetate was added and shaked for 60 sec and liquid phase was transferred to the second separating funnel and extraction procedure was repeated by adding ethyl acetate, then extraction yield was mixed with phosphate buffer. The absorbance measured by spectrophotometer at wave length of 254 nm (Berrios *et al.*, 2004).

Test of capacity to produce cytokinin: Endophytic bacteria were innoculated with Nutrient Broth media and shaked for 72 h, then it was centrifuged, supernatan was extracted with 80% MeOH (Metil Alcohol) for 48 h, then filtrate was evaporated with vaccum until reached liquid phase, then PVP was added and filtered, extracted with ethyl acetate. Filtrate in liquid phase was filtered with Dowex 50Wx4, the elution with NH_4OH 5 N, so that eluate ammonium was produced and then it was measured with

High Performance Liquid Chromatography (Rivier and Crozier, 1987).

Morphology and biochemical test of endophytic bacteria: The morphology observation of five endophytic bacteria included bacteria colony color, colony shape, elevation (perpective from side), edge shape and surface texture (Hadioetomo, 1993). Morphological observation on bacteria cell included cel shape and gram staining (Schaad *et al*., 2001). Biochemical test of isolates was carried out by using microbe kits (MacFaddin, 1979).

Identification of endophytic bacteria based on 16S rRNA partial sequencing: DNA of endophytic bacteria was amplified by using primer 27F and 1492R. DNA sequencing of amplified was carried out at 1st BASE laboratory. Sequencing result data was matched with NCBI Gene Bank data using BLAST where it can be accesed on http://www.ncbi.nlm.nih.gov/.

Obervation with Scanning Electron Microscopy (SEM): To determine the ability of endophytic bacteria to penetrate into plant tissue, suspension of endophytic bacteria was innoculated on rubber planted produced by microcutting technique developed by Indonesian Biotechnology Research Institute for Estate Crops. After 24 h, plantlet was cut and it was prepared with coating equipment and then it was layered with gold and radiated with neutron, then it was put in Scanning Electron Microscopy (SEM) and photograph was selected.

RESULTS AND DISCUSSION

One hundred and seventeen (117) endophytic bacteria were found from isolation of endophytic bacteria originated from rubber plants. Hypersensitive response test was used by using tobbaco to determine whether endophytic bacteria have potential as pathogen for plant. Suspension of endophtyic bacteria was injected to tobbaco (*Nicotiana tabacum* L.) leaf, after 48 h if there was no leaf necrosis, the bacteria were not pathogen for plant. In this experiment, it was found that 71 endophytic have not potential as pathogen to plant.

The next test was Hemolysis test to determine their potency as pathogen for human and animal. Endophytic bacteria were grown in Blood Agar mixed with blood of sheep to make concentration of 5% media, then it was incubated for 18-24 h at room temperature. The presence of clear zone surrounding colony indicated bacteria were categorized as pathogen for human and animal. Hemolysis test resulted in negative result for 55 endophytic bacteria or they were not pathogen for human and animal.

The next step was to test the influence of 55 endophytic bacteria on germination and growth of Ciherang rice seeds. Five endophytic bacteria that resulted the best germination and growth compared with control were chosen after 5 days of incubation. Based on scoring, endophytic bacteria with code KPD6, KPA32, LPD74, LPD76 and KPA38 resulted in best germination and growth. Scoring included germinating rate, length of leaf, length of root, where highest value of parameter had highest score, then the score was summed up. The highest score among 135 incubated seed rice was found on KPD6 with germinating rate 100%, length of leaf and root were 4.64 and 4.28 cm, respectively. The five endophytic bacteria were chosen becuse they had the best effect on germinating and growth compared with other 50 endophytic bacteria and control (Table 1). In control, the germinating rate was 89.33% while the leaf and root length were 2.18 and 3.37 cm, respectively. While, the remaining 50 isolates resulted in germinating rate of 91.67% with leaf and root length of 2.36 and 4.13 cm, respectively.

The morphological observation of endophytic bacteria was carried out by observing color, shape, elevation (from side perspective), edge shape and surface texture of colony. The colony morphology observed of 5 endophytic bacteria isolates showed that colony color was white except LPD74 was yellow, colony shape of all bacteria were circular, elevation was raised except LPD74 was convex, shape of margin was entire but KPA32 and KPA38 were undulate, surface texture of bacteria was smooth shiny except KPA32 that was dry.

The cell morphology of five endophytic bacteria isolates showed that gram staining was negative except KPD6 and LPD74 that were positive and shape of cell was bacil except LPD74 that was coccus, also sizes of each isolate varied.

Biochemical tests for five endophytic bacteria isolates showed positive for catalase, oxidase, motility, nitrate, glucose and VP tests. While, negative result was found for ornithine and arginine. The other biochemical tests varied.

Table 1: Growth and germination scoring of rice seed innoculted with 5 endophytic bacteria isolates

Isolate code	Average length of shoot (cm)	Score	Average length of root (cm)	Score	Average germination rate (%)	Score	Total of score
KPD6	4.64	55	4.28	28	100.00	52	135
LPD74	2.76	40	5.87	52	96.67	42	134
KPA32	4.34	52	4.46	35	96.67	42	129
KPA38	2.56	35	5.54	50	95.00	38	123
LPD76	2.36	26	5.62	51	98.33	46	123

Table 2: Morphological characters and biochemistry test of 5 endophytic bacteria isolates

	Isolates				
Characters	KPD6	KPA32	LPD74	LPD76	KPA38
Colony morphology					
Color of colony	White	White	Yellow	White	White
Shape of colony	Circular	Circular	Circular	Circular	Circular
Elevation	Raised	Raised	Convex	Raised	Raised
Shape of margin	Entire	Undulate	Entire	Entire	Undulate
Surface	Smooth	Dry	Smooth	Smooth	Smooth
Texture	Shiny		Shiny	Shiny	Shiny
Cell morphology					
Gram staining	+	-	+	-	-
Shape of cell	Bacil	Bacil	Coccus	Bacil	Bacil
Size (um)	0.5×2	0.25×0.5	0.5×0.5	0.25×1	0.25×1
Biochemical test					
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Nitrate	+	+	+	+	+
Lysine	-	-	-	+	-
Ornithine	-	-	-	-	-
H_2S	-	-	-	+	-
Glucose	+	+	+	+	+
Mannitol	-	+	+	-	+
Xylose	-	+	+	+	+
ONPG	-	+	+	+	-
Indole	-	-	-	+	+
Urease	+	+	-	+	+
VP	+	+	+	+	+
Citrate	-	+	-	+	+
TDA	-	-	-	-	+
Gelatin	+	-	-	+	
Malonate	-	-	-	+	-
Inositol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Rhamnose	-	+	+	+	+
Sucrose	-	+	+	+	-
Lactose	-	+	+	-	-
Arabinose	-	+	+	+	+
Adonitol	-	-	+	+	+
Raffinose	-	-	+	+	-
Salicin	-	-	+	+	-
Arginine	-	-	-	-	-

Table 3: Ability of five endophytic bacteria to fix N_2 measured with ARA method, produce IAA, gibberellin and cytokinin (zeatin and kinetin) hormones

				Cytokinin	
Endophytic bacteria	ARA (nmole C_2H_4/µL/h)	IAA (µg mL^{-1})	Gibberellin (µg mL^{-1})	Zeatin (µg mL^{-1})	Kinetin (µg mL^{-1})
B. cereus	4.230	111.00	27.00	0.020	0.015
P. aeruginosa	3.705	53.67	23.63	0.025	0.029
B.paracongmeratum	3.396	119.00	7.50	0.012	0.004
Bacterium sp.	2.843	28.17	60.00	0.018	0.016
P. vermicola	3.482	114.83	20.75	0.020	0.012

The result of morphology of colony, bacteria cell and the biochemical test for five isolates using microbe kit is presented in Table 2.

Identification of endophytic bacteria based on partial sequencing 16S rRNA showed that isolate of KPD6, KPA32, LPD74, LPD76 and KPA38 were known as *Bacillus cereus, Pseudomonas aeruginosa, Brachybacterium paraconglomeratum, Bacterium* and *Providencia vermicola.*

The capacity of five bacteria to fix N_2, produce hormone of Indole Acetic Acid (IAA), gibberellin and cytokinin is shown in Table 3. The capacity to fix N_2 was estimated by using ARA method where, the capacity to reduce of acethylene by measuring ethylene produced. The ethylene produced was 28.43-42.30 nmol μL^{-1} h^{-1} where, *Bacillus cereus* was the highest.

The capacity to produce IAA hormone was 28.167-119 µg mL^{-1} where *Brachybacterium paraconglomeratum* produced the highest while, gibberellin production was 7.5-60 µg mL^{-1} where production the highest giberellin while cytokinin (zeatin) production was 0.012-0.025 µg mL^{-1} and cytokinin

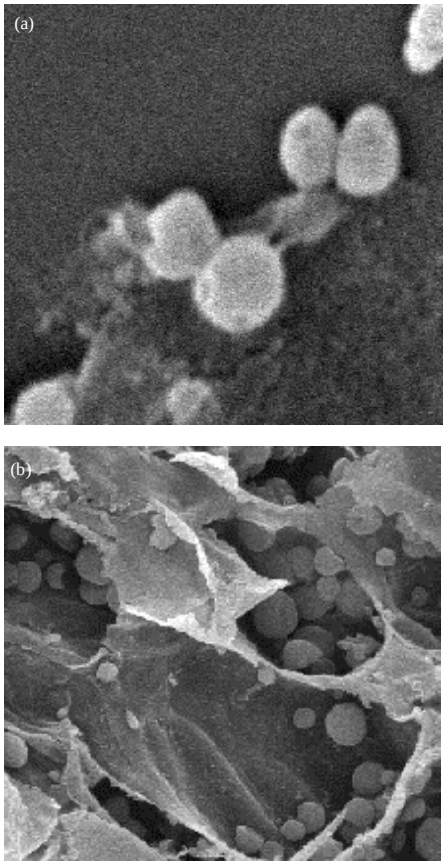

Fig. 1(a-b): Scanning Electron Microscopy photograph of *Brachybacterium paraconglomeratum* on endophytic bacteria (a) Nutrient Broth media (10.000x) and (b) Rubber planlet (750x)

(kinetin) was 0.004-0.029 μg mL^{-1} where *Pseudomonas aeruginosa* produced the highest cytokinin.

Growth promoting hormone is endogen and exogen substances that anable to change plant growth. IAA function was to stimulate cell enlargement, root growth, flowering and prevent fruit drop. Gibberellin promoted the flower initiation and make flowering uniform, while cytokinin was stimulating cell division and mitosis differentiation (Wattimena, 1987).

One of identified bacteria was *Pseudomonas* and it was also found by Mendes *et al.* (2007) that demonstrated endophytic bacteria found in root and stem of sugarcane produced growth promoting hormone such as Indole Acetic Acid (IAA). Furthermore, Mendes *et al.* (2007) also found bacteria of *Burkholderia*, *Pantoea* and *Microbacterium*. Malik *et al.* (1997) found that *Pseudomonas* 96-51 isolated from paddy rhizosphere could produce IAA as much as 35.7 μg mL^{-1} while in this experiment, *Pseudomonas aeruginosa* could produce higher IAA that was 53.67 μg mL^{-1}.

Futhermore, Scanning Electron Microscopy (SEM) was carried on endophytic bacteria of *Brachybacterium paraconglomeratum* innoculated on rubber plantlet produced by micro cutting technique, (Fig. 1). Figure 1 shows that endophytic bacteria in nutrient broth media is the same as endophytic bacteria that penetrated into planlet tissue and this demonstate that the endophytic bacteria is alive in plant tissue.

CONCLUSION

One hundred and seventeen endophytic bacteria were found from isolation of endophytic bacteria originated from rubber plants. Hypersensitive response test found that 71 endophytic have not potential as pathogen to plant. Hemylosis test resulted in negative result for 55 endophytic bacteria. Five endophytic bacteria resulted the best germination and growth compared with control.

The capacity to fix N_2 estimated by using ARA method was 28.43-42.30 nmol μL h^{-1}. The capacity to produce IAA hormone was 28.167-119 μg mL^{-1} while gibberellin produced was 7.5-60 μg mL^{-1} while cytokinin (zeatin) produce was 0.012-0.025 μg mL^{-1} and cytokinin (kinetin) was 0.004-0.029 μg mL^{-1}.

Identification of 5 endophytic bacteria based on partial sequencing 16S rRNA showed as *Bacillus cereus, Pseudomonas aeruginosa, Brachybacterium paraconglomeratum, Bacterium* and *Providencia vermicola.* Scanning Electron Microscopy (SEM) showed that endophytic bacteria penetrate into rubber planlet tissue.

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