GROWTH-PROMOTING PROPERTIES OF BACTERIA ISOLATED FROM DIPTEROCARP PLANTS OF ACIDIC LOWLAND TROPICAL PEAT FOREST IN CENTRAL KALIMANTAN, INDONESIA

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ABSTRACT

Dipterocarpaceae is a group of native, predominant trees of Indonesian lowland tropical forests. It is now under a serious threat of extinction due to continual forest degradations. Although dipterocarps have been used for reforestation, in the case of peat forests, transplanted seedlings typically suffer low survival rates due to acidic soil, low nutrient content, and the presence of toxic elements. To produce robust seedlings, many studies have suggested an inclusion of plant growth-promoting rhizobacteria (PGPR). However, little or no studies have been done to PGPR of dipterocarps. This study is a part of our wider effort to search for potential PGPR for bio-reforestation in degraded lands in Indonesia. We characterized PGPR of dipterocarps for their capacities to solubilize P, fix N_2, and/or help ectomycorrhization *in vitro*. Bacteria were isolated from *Shorea teysmanniana*, *S. parviflora*, *S. belangeran*, *S. stenoptera*, *Dipterocarpus* sp., and *Hopea* sp. of acidic, lowland peat swamp forest in Central Kalimantan. A total of 71 bacterial strains from rhizoplane and rhizosphere were isolated in an N-free Winogradsky's soft gel medium. While most bacteria possessed PGPR traits *in vitro*, 14 showed the best PGPR efficiency. The best P solubilizers were *Erwinia* spp. CK23, CK24 and CK10, *Roseateles* sp. CK15, *Rhizobium* sp. CK19, *Burkholderia* sp. CK52, NI CK36, NI CK42, NI CK53, and NI CK54. The highest N_2 fixers were *Azospirillum* sp. CK26, *Burkholderia* sp. CK32, and NI CK4. A *Chromobacterium* sp. CK8 was the most potential ectomycorrhization helper of *Laccaria* sp. We demonstrated that Dipterocarpaceae harbored diverse bacterial genera and potential PGPR candidates which may be utilized as growth promoters for preparation of robust seedlings for bio-reforestation of degraded lands.

Keywords: PGPR, N_2 fixation, P solubilization, ectomycorrhization, *Erwinia* spp., *Azospirillum* sp., *Chromobacterium* sp., bioreforestation

I. INTRODUCTION

Lowland peat swamp forests in Central Kalimantan, Indonesia, are heavily disturbed due to excessive logging, poor management and land conversion. During 1985-2001 period, more than 56% or over 29,000 km^2 of protected lowland forests of Kalimantan have been degraded (Curran *et al.*, 2004). The continual

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destruction consequently threats the existence of the most important tree family, Dipterocarpaceae, that predominates this habitat and is native to Indonesian forests. On the other hand, natural regeneration of dipterocarps is low, hence these degraded areas are dominated by fern, scrub or sedge, such as *Imperata cylindrica* (alang-alang), *Melastoma malabathricum,* and *Xyris complanata*. The difficulties of tree regeneration in these areas are attributable to typically acidic soil, the presence of toxic elements, and low nutrient content. Only 0.1% of the total P present is available to the plants because of its chemical fixation and low solubility (Tilak *et al*., 2005) and, especially at our sampling site, the total nitrogen content was lower than 0.9%. In addition, high water table, lack of shading, and frequent outbreaks of fire also hamper tree growth, thus transplanted saplings into these degraded areas typically suffer low survival rates. The usual practice of reforestation based solely on transplants with standard chemical inputs is often ineffective and ecologically unsustainable.

Under such conditions, microorganisms offer a biological source of nutrients. The term plant growth-promoting rhizobacteria (PGPR) is used in describing soil rhizobacteria that are capable of stimulating plant growth (Glick, 1995; Kokalis-Burelle *et al.,* 2006) by various mechanisms. Plant growth-promoting rhizobacteria have gained wide attention due to their beneficial effects on plant growth. The effects may involve the provision of available phosphorus, fixation of nitrogen, sequestration of iron by siderophores, production of plant hormones, lowering of plant ethylene levels, production of antibiotics, induction of systemic resistance against pathogenic bacteria (Glick, 1995; Lucy *et al*., 2004) and/or helping the establishment of ectomycorrhiza (Garbaye, 1994). Therefore, many attempts have been made to use PGPR to improve the plant growth of various species, but most of these have been towards growth of annual crops. Recently, there has been an increasing interest in the use of PGPR for the improvement of tree seedlings in the nursery for forestry management (Chanway, 1997; Garcia *et al*., 2004). Reports of PGPR effects on the conifers (*Pinus, Picea, Tsuga,* and *Pseudotsuga*) have been documented (Chanway, 1997). However, no studies have been done on PGPR for tropical tree species, especially Dipterocarpaceae, to the best of our knowledge.

Dipterocarpaceae is also an ectomycorrhiza-dependent tree (Turjaman *et al.,* 2005) and certain bacteria have been reported for their ability to help the establishment of mycorrhizal formation (Garbaye, 1994). Mycorrhiza is a symbiotic relationship between plant roots and beneficial fungi that may increase stress tolerance, improve nutrient cycling, assist in P uptake, control phytopathogens, synthesize phytohormones, and improve soil structure (Jindal *et al.,* 1995). A study by Kashyap *et al.* (2004) suggested that a dual inoculation of *Morus alba* (Moraceae) with arbuscular mycorrhizal fungi and *Azotobacter*, and an addition of indole butyric acid significantly enhanced the survival percentage of seedlings from 25 to 50% under salt stress condition.

For this reason, we investigated rhizobacteria of dipterocarp saplings and seedlings growing in acidic, lowland peat soil in Central Kalimantan. We hypothesized

that certain bacteria that live in close vicinity with Dipterocarpaceae also possess specific PGPR traits that can be utilized for the production of robust seedlings that can survive after outplanting into such degraded lands. The objectives of this study were: (1) to trap rhizobacteria from peat-adaptable dipterocarp tree saplings and seedlings; (2) to screen P solubilizing, N_2 fixing, and/or ectomycorrhization helper bacteria (ECMHB) *in vitro.*

The information provided here is a part of our wider efforts to restore degraded forest areas in Indonesia by using local bio-potent PGPR for the growth promotion of local peat-adaptable Dipterocarpaceae.

II. MATERIALS AND METHODS

A. Sampling Site Conditions

Bacteria were isolated from two localities: Nyaru Menteng arboretum and nursery of Center for International Cooperation in Sustainable Management of Tropical Peatland (CIMTROP), the University of Palangkaraya in Central Kalimantan, Indonesia (Figure 1). Nyaru Menteng arboretum is located 28 km outside Palangkaraya city at 2°43'49"S; 111°38'54"E and is protected from deforestation activities that occur in many forest areas in Central Kalimantan. The area covers 62.5 ha of lowland peat swamp forest ecosystem grown with a total of 64 local peat-adapted trees and a population density of approximately 1,000 trees per ha including many dipterocarp species (Saribi and Riswan, 1997), and is under the management of the Natural Resources Conservation Institute under the Ministry of Forestry (NRCI-MoF), Indonesia. The average annual rainfall is 3,120 mm with an average of 16 rainy days per month while the average temperature is from 18 to 34°C with a relative humidity being 18% (NRCI-MoF, *pers. comm.*). Eight naturally-emerging dipterocarp saplings of various dipterocarp genera from underneath mature stands were randomly selected. The age of saplings varied, from 6 to 18 months old. The whole saplings were taken out from approximately 20 cm-deep peat soil. The second locality was Nursery of CIMTROP, located in the Palangkaraya city. Three saplings aged

Figure 1. Location of sampling sites in Central Kalimantan, Indonesia (black dot indicates sampling location)

approximately 1, 2.5, and 3 years old were sampled. The soil pH ranged from 3.3 to 4.4 in H_2O.

B. Quantitative Analysis of Peat Chemical Properties at Nyaru Menteng Arboretum Site

Chemical and physical analyses of soil samples were done only towards the peat soil of Nyaru Menteng Arboretum, Central Kalimantan as follows. Peat soil was sampled at two random points from a depth of 0-15 cm in the sampling site in Nyaru Menteng arboretum. As a comparison, two additional samples were randomly taken from a similar depth from a nearby site that had a different soil composition of a mixture of peat and mineral soils. Sieved (2 mm), air-dried samples were used for the chemical analysis. Ten grams of the sample were measured for pH after 1 h shaking with distilled water or one Molar KCl solution. Analyses of other properties were carried out by standard methods as follows: total organic C by Walkley-Black C method (Walkey and Black, 1934), total N by Kjeldhal method (Bremner and Mulvaney, 1982), and available P by Olsen method (Olsen and Sommers, 1982). Cation exchange capacity (CEC) was determined by analyzing NH_4-N concentrations. This extract was further used to determine the exchangeable cations (K, Ca, Na, and Mg). Base saturation was calculated by dividing CEC by the sum of exchangeable cations.

C. Bacterial Isolation

Soil attached to the root following vigorous shaking, was regarded as rhizosphere soil for isolation of rhizosphere bacteria. Fibrous roots of 1-cm in length, completely washed and cleaned with sterile water, were used for the sources of rhizoplane bacteria. The root was then incubated for 24 h at 28°C in an N-free, soft gel medium of Winogradsky's mineral mixture, which contained 1% sucrose as the sole carbon source and 0.3% gellan gum as the gel matrix (Hashidoko *et al.*, 2002). This medium is routinely used in our laboratory to trap and preliminarily screen nitrogen-fixing bacteria from the field, although the ability to fix nitrogen must be further proven by an acetylene reduction assay or analysis of *nif* gene. After incubation, the root was removed from the soft gel medium under aseptic condition and the culture was further incubated for 48 h at 28°C in the dark. The rhizosphere peat soil was vortexed in 10 ml of 0.7% NaCl as saline buffer solution to prevent osmotic pressure shock and to disperse bacterial cells evenly in the solution, after which one loop-full of soil slurry was stabbed into the same medium. One loop-full of emerging bacteria from the soft gel medium was, after a 3-day-incubation at 28°C in the dark, transferred to one plate containing 10 ml of modified Winogradsky's (MW) agar medium. This MW also contained 0.005% yeast extract as an extra-component and 2% agar. Each distinguishable colony was purified on the same plate medium. A total of 71 isolates from the rhizoplane and rhizosphere were selected based on different colony types (color, thickness, turbidity, stickiness, and shape) on the MW agar. Finally, each single colony was grown and maintained in

the gellan gum-Winogradsky's medium at 28°C for further use and stored in 10% glycerol at -84°C.

D. Identification of Bacteria based on Sequence Determination of 16S rRNA Gene Region

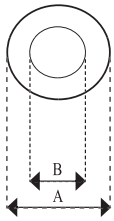
Molecular identification of bacteria based on partial 16S rRNA gene sequencing was done according to a routine protocol in our laboratory as follows. Total DNA of the isolates was prepared using a commercial DNA-preparation kit (Isoplant II®, Wako Pure Chemical Industries Co. Ltd., Osaka, Japan) as the polymerase chain reaction (PCR) template. The 16S rRNA gene region was amplified by PCR using HotStarTaq™ kit (Qiagen, Hilden, Germany), and the universal forward (27F) and reverse (1525R) primers (Weisburg *et al*., 1991) under reaction conditions of hot starting at 95°C for 15 min, 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and final annealing at 72°C for 10 min.

The amplified product of approximately 1.5 kb in size was directly sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with four choices of universal primers: 1112F, 926F, 1080RM, and/or 518R under the conditions of 1 cycle of 96°C for 5 min and 25 cycles of 96°C for 30 s, 50°C for 15 s, 60°C for 4 min using ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, California, USA). Sequence homology was sought for using a BLASTN online DNA database from the National Center for Biotechnology Information (NCBI). Each identified strain was submitted to the GenBank database to obtain its accession number.

E. Characterization of P-Solubilization and Mineralization Trait

Because peat swamp contains inorganic and organic phosphorus, both P sources were used for screening the P solubilizer on a plate assay using a National Botanical Research Institute`s Phosphate medium (NBRIP) containing L^{-1}: glucose, 10 g: $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g (Nautiyal, 1999) with different sources of P. (1) $Ca_3(PO_4)_2$ is a common inorganic P source used for P solubilization screening. Thus, NBRIP medium with $Ca_3(PO_4)_2$ solidified with 1.5% agar and with the pH adjusted to 7.0 before sterilization, was used as the initial screening medium for all 71 strains. The plates were incubated at 28°C in the dark and P solubilization, shown by clear zones on the medium, was measured at 4, 6, and 8 days after inoculation. (2) Secondary screening for strains that had a solubilization index (SI) $\geq$ 3, at 8 days after inoculation used different sources of both inorganic and organic P modified with lower pH, because the bacteria were acidophilic. Then, only 10% N source was used, because the bacteria were originally trapped using a nitrogen-free medium. The P sources and pH of the three media were (1) $Ca_3(PO_4)_2$ at pH 4.5; (2) $C_6H_6(OPO_3H_2)_6$ (phytic acid) at pH 6 to test the hypothesis that peat soil contains a large amount of phytic acid and thus the bacteria in the soil were able to mineralize the organic phosphate phytic

acid; and (3) $AlPO_4$ (+ $CaCl_2$) at pH 4.3. $CaCl_2$ served as a transporter agent to assist solubilization of $AlPO_4$ by bacteria (Rodríguez and Fraga, 1999) in the NBRIP medium. In the initial screening, three strains per plate were inoculated to the test media in triplicate by sterile toothpicks, while in the secondary screening, three to four replications for one strain per plate were used. The solubilization index (Figure 2) was calculated as the ratio of total diameter (colony + halo zone) to diameter of the colony (Premono *et al.*, 1996). Data were subjected to statistical analysis.



Phosphate Solubilization Index = A/B

A = total diameter (colony + halo zone)
B = diameter colony

Figure 2. Calculation of phosphate solubilization index

F. Characterization of N-Fixation Trait

In nitrogen fixation, the nitrogenase enzyme complex that converts nitrogen to ammonia, also mediates the reduction of acetylene (C_2H_2) into ethylene (C_2H_4). Here, the acetylene reduction assay (ARA) was used as a rapid and simple method to indirectly screen nitrogen-fixing bacteria. Bacteria were cultured in 30 ml Screw Cap Septum (SCS) vials (Pierce, Biotechnology, Inc., Rockford, USA) containing a 10 ml nitrogen-free soft gel medium of Winogradsky's mineral mixture which contained 1% sucrose as the sole carbon source and solidified with 0.3% gellan gum, similar to the one used earlier. In addition, 150 mg of absorbent cotton was supplied as a tridimensional surface for bacterial attachment and the medium was autoclaved at 121°C, 2 atm for 20 min. One loop-full of bacteria was inoculated and incubated for 2 weeks at 28°C in the dark, then 1.5 ml of acetylene gas was injected into the SCS vial, vortexed, and the culture was further incubated for 3 days. Two screening steps were applied. First, all of the 71 strains, non-replicated, were rapidly screened for N_2 fixing potential by measuring ethylene production 3 days after acetylene injection. Second, strains showing positive acetylene reduction were again assayed in triplicates to further quantify the ethylene produced over time at 3, 6, and 8 days after acetylene injection. A free-living nitrogen-fixing bacterium, *Beijerinckia indica* subsp. *indica* IFO 3744 (Beking, 1984), was included in the assay as reference of the positive control.

For ethylene detection, 25μl gas was taken from each SCS-vial and injected to a gas chromatograph (GC), G-5000 (Hitachi, Japan) equipped with a glass capillary column CP-Pora PLOT U (Chrompack, The Netherlands), at 50 KPa in the injector. In addition, the bacterial population was also measured by optical

density at 665 nm before and after the ARA analysis using LS-PLATE Manager 2000 Ver.1.01 (Wako Pure Chemical Industries Ltd., Osaka, Japan). Data were subjected to statistical analysis.

G. Characterization of Ectomycorrhization Helper Trait

Because bacteria can stimulate, inhibit, or have no effect on the growth of mycorrhizal fungus (Bowen and Theodorou, 1979), a dual plate assay was used to investigate the interaction of an ectomycorrhizal fungus (ECM), *Laccaria* sp. with 71 bacteria. Two types of agar media and three models of dual inoculations were applied. First, initial screening used MW agar medium as it was used earlier for isolation and purification of the bacteria. A single 0.5 cm diameter plug of ECM fungus placed at the center of the plate was incubated for 7 days at 28°C in the dark, followed by stabbing eight strains of bacteria twice by sterile toothpicks around the colony, at approximately 2.0 and 3.0 cm from the center of the plate (Figure 3a). The blank was a single 0.5 cm plug of ECM fungus without bacteria. From this result, bacteria that inhibited or promoted growth of the fungus relative to the control were further screened for dual culture assay using two media: MW and 2% potato dextrose agar (PDA). In this secondary screening, bacterium was streaked along the center of the medium, dividing the plate into two halves and at the same time, two fungal plugs of 0.5 cm diameter were placed on the medium (Figure 3b). Microscopic observation was done to visualize the morphology of hyphal growth, when growing mycelia of the blank (without bacterium) from both edges collided. Bacteria that promoted the growth of the fungus were regarded as the ECMHB. To quantify the radial fungal growth in the presence of promoting- or inhibiting-bacteria, selected bacteria were co-inoculated with the fungus *Laccaria* in triplicate. A 0.5 cm mycelial plug was placed at the center of the plate containing 15 ml 2% PDA and was incubated at 28 °C in the dark for 5 days. Then, from the 0.5 cm mycelial plug, bacteria were inoculated at four equidistant points (Figure 3c). Radial growth of the fungus after 8 and 11 days of co-inoculation was measured. The data were subjected to statistical analysis.

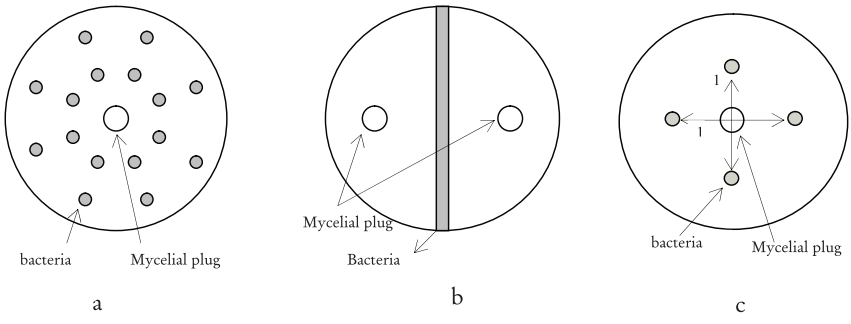


Figure 3. Dual assay plate of mycorrhization helper bacteria and *Laccaria* sp.

Qualitative initial screening with eight different bacterial strains stabbed twice around the colony, and secondary screening using one bacterial strain streaked in the center. Quantitative bacterial strains were co-inoculated at four equidistant points from the 0.5 cm diameter mycelia plug. Radial growth was mycelia (cm) growing towards the bacterial colony.

H. Statistical Analysis

All quantitative data were subjected to one-way analysis of variance using SPSS® version 10.0 (SPSS Inc.) with the mean values compared using the Fisher's LSD (least significant difference) ($p = 0.05$).

III. RESULTS AND DISCUSSION

A. Results

1. Peat chemical properties at Nyaru Menteng arboretum site

The two different types of samples showed different chemical properties but both samples had a low pH which ranged from 2.50 to 3.19 in KCl and from 3.40 to 3.90 in H_2O (Table 1). The C/N ratio was within a normal range between 50 and 100% (Inubushi *et al*., 2003) although samples from a mixture group showed a much lower ratio than the pure peat group samples. Total organic C was within a normal range (50 - 60%) in the pure group, i.e. 52.2 - 54.3%. The low C in the second group confirmed that the peat was actually a mixture with mineral soil. The total N was low in both types of peat because the normal total N in peat soil was reported to be more than 1% (Inubushi *et al*., 2003; Takakai *et al.,* 2006). Available P contents were variable in both groups ranged from 9.2 to 30.9 ppm. Pure peat retained higher contents of exchangeable cations, CEC, Al, proton, and water content (Table 1).

Table 1. Quantitative data of peat chemical properties from four random spots at the Nyaru Menteng sampling sites, Central Kalimantan

Chemical properties/type of peat	Pure peat		Peat and mineral soil mixture	
	1	2	1	2
Exchangeable cations (me/100 g):				
Ca	2.27	1.74	1.63	1.27
Mg	1.38	0.82	0.86	0.70
Na	0.50	0.61	0.42	0.48
K	0.34	0.49	0.30	0.36
Cation Exchange Capacity (me/100 g)	60.8	49.3	18.3	23.1
Base Saturation (%)	7.40	7.40	17.5	12.2
Al (me/100 g)	1.04	3.02	1.68	1.54
H (me/100 g)	0.69	0.87	0.74	0.76
Water content (%)	76.6	89.4	15.5	15.1
pH 1:1				
H_2O	3.50	3.40	3.90	3.70
KCl	2.60	2.50	3.19	2.70
C-organic (%)	54.3	52.2	19.5	19.5
N-total (%)-Kjeldhal	0.82	0.76	0.37	0.39
C/N (%)	66.2	68.7	51.5	50.1
P-Bray I (ppm)	30.9	18.4	9.2	10.3

Notes: 1 and 2 show number of peat samples taken randomly from pure peat, and peat and mineral soil mixture for analysis

2. Identification of bacteria based on 16S rRNA gene region

Based on 16S rRNA partial gene sequencing homology, we identified 56 bacteria from the total of 71 microbial isolates from the rhizosphere and rhizoplane of dipterocarp saplings and seedlings (Table 2). These bacteria belonged to α-, β-, or γ-subclass of Proteobacteria and a few were bacilli. Rhizoplanes were inhabited by Gram-negative bacteria, except one strain, *Bacillus* sp. CK39 that was found colonizing the rhizoplane of *S. stenoptera* grown from a seed in a pot under green house conditions. Four other Gram-positive bacteria were isolated from rhizosphere soil. In total, the rhizoplane and rhizosphere were inhabited by 13 and 9 different genera, respectively. Genera *Burkholderia, Frateuria, Klebsiella, Rhizobium, Enterobacter,* and *Bacillus* could be found in both extracts with various frequencies of occurrence. Three *Erwinia* and two *Sphingomonas* strains were found only on the rhizoplane. The genus *Burkholderia* was the most frequently isolated bacterium, 22 of 56 identified from both isolation sources. In particular, *S. teysmanniana* aged approximately 6 month and 1 year harbored the most diverse genera on their rhizoplane: *Klebsiella, Rhizobium, Sphingomonas, Azospirillum, Rhizobium, Burkholderia,* and *Pandoraea*. The 15 strains that could not be identified by our routine protocols may belong to subclasses other than proteobacteria and

bacilli. Microscopic observation showed that these unidentified microorganisms were mostly Gram-negative and of small-size cocci, less than 1.5 μm. Further research is still ongoing to reveal their identity.

Table 2. Bacterial strains isolated from species of dipterocarp saplings and seedlings

a. Nyaru Menteng Arboretum

Host	Location	Age	Identification (GenBank Accession No.)	Base length (Identity %)	Close match with NCBI database
Hopea sp.	Rhizoplane	~ 1y	*Frateuria* sp. CK1 (DQ195883)	1267 (97)	*Frateuria* sp. EC-K130
			Frateuria sp. CK2 (DQ195891)	700 (98)	*F. aurantia* strain IFO13333
			NI CK3		
			Frateuria sp. CK14 (DQ195878)	1507 (96)	*F. aurantia* strain IFO13329
			Roseateles sp. CK15 (DQ195879)	460 (99)	*R. depolymerans* 61B2
			NI CK16		(DSM11814)
	Rhizosphere	~ 1y	*Klebsiella* sp. CK40 (DQ195899)	1457 (94)	*K. planticola* ATCC 33531T
			Bacillus sp. CK41 (DQ195900) NI	1285 (97)	*B. cereus* strain BGSC
			CK42		6A5rrnM operon
			Bacillus cereus. CK50 (DQ195908)	1456 (97)	*B. cereus*
			Burkholderia sp. CK51 (DQ195909)	923 (95)	*B. tropica* strain MTo-293
			Burkholderia sp. CK52 (DQ195910)	401 (98)	*Bukholderia* sp.
Shorea parviflora	Rhizoplane	~6m	*Enterobacter* sp. CK10 (DQ195874)	873 (98)	*E. kobei* strain CIP105566
			Burkholderia sp. CK11 (DQ195875)	415 (99)	*Burkholderia* sp. FL44
			Erwinia sp. CK12 (DQ195876)	426 (99)	*E. persicina* strain LPPA373
			Enterobacter sp. CK13 (DQ195877)	1178 (97)	*Enterobacter* sp. M9kro15
		~1.5y	*Frateuria* sp. CK7 (DQ195925)	630 (97)	*F. aurantia* IFO13333
			Chromobacterium sp. CK8	374 (94)	*Chromobacterium* sp. 71
			(DQ195926)		
			Burkholderia sp. CK9 (DQ195927)	373 (99)	*B. cepacia* ATCC 53130
	Rhizosphere	~6m	*Rhizobium* sp. CK48 DQ195906	906 (99)	*R. rhizogenes* strain 163C
			Frateuria sp. CK49 DQ195907	405 (96)	*Frateuria* sp. EC-K130
		~1.5y	*Burkholderia* sp. CK46 DQ195904	1280 (97)	*B. gladioli*
			Burkholderia sp. CK47 DQ195905	430 (99)	*Burkholderia* sp. 14
Shorea teysmanniana	Rhizoplane	~6m	*Klebsiella* sp. CK17 (DQ195880)	1336 (99)	*Klebsiella* sp. 141
			Erwinia sp. CK18 (DQ195881)	1388 (98)	*E. cypripedii*
			Rhizobium sp. CK19 (DQ195882)	693 (96)	*R. tropici* UPRM8033
		~1y	*Sphingomonas* sp. CK25 (DQ195887)	959 (98)	*Sphingomonas* sp. IW3
			Azospirillum sp. CK26	1385 (96)	*A. amazonense* DSM2787
			Rhizobium sp. CK27 (DQ195888)	410 (98)	*Rhizobium* sp. RM1-2001
			Burkholderia sp. CK28 (DQ195889)	348 (91)	*B. tropicalis* strain PPe6
	Rhizosphere	~6m	NI CK53		
			NI CK54		
			NI CK55		
		~1y	*Burkholderia* sp. CK59 (DQ195914)	1471 (97)	*B. phenazinium*
			Pandoraea sp. CK60 (DQ195915)	403 (98)	*Pandoraea* sp. G3307
			NI CK61		
			Burkholderia sp. CK62	1162 (98)	*B. tropicalis* strain LM1-376.8

Host	Location	Age	Identification (GenBank Accession No.)	Base length (Identity %)	Close match with NCBI database
Diptero-carpus sp.	Rhizoplane	~1y	*Sphingomonas* sp. CK20 (DQ195884)	323 (98)	*Sphingomonas* sp. SB3
			NI CK21		
			NI CK22		
			Erwinia sp. CK23 (DQ195885)	1360 (97)	*E. cypripedii*
			Erwinia sp. CK24 (DQ195886)	1372 (98)	*E. cypripedii*
			Stenotrophomonas sp. CK34 (DQ195895)	945 (94)	*S. maltophilia* strain AUX077
			NI CK35		
			NI CK36		
	Rhizosphere	~1y	*Burkholderia* sp. CK56 (DQ195911)	417 (100)	*Burkholderia* sp. IBRC204
			Paenibacillus sp. CK57 (DQ195912)	941 (99)	*Paenibacillus* sp. tjsxy2003-j-67
			Paenibacillus sp. CK58 (DQ195913)	394 (98)	*Paenibacillus* sp. tjsxy2003-e-4
			Burkholderia sp. CK66 (DQ195921)	1478 (98)	*B. tropica* strain MTo-672
			Serratia sp. CK67	261 (100)	*S. marcescens* isolate DR.Y10
			Burkholderia sp. CK68 (DQ195918)	376 (100)	*B. cepacia* ATCC 53130

b. Palangkaraya University Nursery

Host	Location	Age	Tentative identification (GenBank Accession No.)	Base length (Identity %)	Close match with NCBI database
Shorea belangeran	Rhizoplane	~1y	NI CK4		
			NI CK5		
			Klebsiella sp. CK6 (DQ195920)	1265 (97)	*Klebsiella* sp. I-F7
		~3y	*Frateuria* sp. CK29 (DQ195890)	385 (96)	*Frateuria* sp. EC-K130
			Burkholderia sp. CK30 (DQ195892)	1190 (99)	*B. cepacia* TC621
			Burkholderia sp. CK31 (DQ195893)	612 (97)	*Burkholderia* sp. FL44
			Burkholderia sp. CK32 (DQ195894)	393 (99)	*B. cepacia* ATCC53130
			Burkholderia sp. CK33 (DQ205479)	933 (98)	*B. cepacia* ATCC53796
	Rhizosphere	~1y	*Burkholderia* sp. CK43 (DQ195901)	933 (99)	*B. cepacia* ATCC17762
			Burkholderia sp. CK44 (DQ195902)	1165 (98)	*B. tropica* strain MTo-293
			Burkholderia sp. CK45 (DQ195903)	1268 (95)	*B. cepacia* ATCC17460
		~3y	*Pseudomonas* sp. CK63 (DQ195916)	1487 (99)	*Pseudomonas* sp. KIE 171-B
			Enterobacter sp. CK64 (DQ195917)	402 (94)	*E. sakazakii* strain ATCC 51329
			NI CK65		
Shorea stenoptera	Rhizoplane	~2.5 y	*Burkholderia* sp. CK37 (DQ195896)	1417 (98)	*B. cepacia* ATCC49709
			Burkholderia sp. CK38 (DQ195897)	1387 (100)	*B. cepacia* ATCC49709
			Bacillus sp. CK39 (DQ195898)	488 (99)	*Bacillus* sp. R423
	Rhizosphere	~2.5 y	*Enterobacter* sp. CK69 (DQ195919)	365 (98)	*Enterobacter* sp. strain B5
			NI CK70		
			Burkholderia sp. CK71 (DQ195922)	382 (99)	*B. cepacia* strain ATCC53130

Notes: CK: Central Kalimantan; NI: unidentified microorganisms, mostly Gram-negative, cocci-like and less than 1.5 μm in size; y: year old; M: months old; NCBI: the National Center for Biotechnology Information

3. Phosphate solubilization and mineralization activity

Four days after the inoculation, most strains were able to grow and solubilize inorganic phosphate in the form of $Ca_3(PO_4)_2$ with various indexes of solubilization

(data not presented). Six isolates, *Klebsiella* sp. CK6, *Frateuria* sp. CK7, *Burkholderia* spp. CK32 and CK51, *Stenotrophomonas* sp. CK34, *Enterobacter* sp. CK69, and two unidentified CK35 and CK70 had a solubilization zone the same as the colony diameter (SI = 1.00) for all days observed. Three isolates, *Frateuria* sp. CK14, *Paenibacillus* sp. CK57 and CK58, grew on the medium but showed no solubilization indicating that they used other sources besides P in the medium. Eleven strains, *Sphingomonas* spp. CK20 and CK25, *Rhizobium* sp. CK27, *Burkholderia* spp. CK30, CK33, CK37, CK38, and CK71, and *Bacillus* spp. CK39 and CK50, were unable to grow in NBRIP's medium at pH 7.0. On the other hand, the highest solubilization indexes (SI) were 5.67, 6.64, and 6.08 for 4, 6, and 8 days after inoculation, respectively (Table 3). Whilst the least efficient SI was 1.0 showing that the solubilization zone was as wide as the colony diameter. From this initial screening, ten bacterial strains having the most efficient P solubilizers on 4, 6 and 8 days after inoculations were selected for further screening (Table 3): *Erwinia* spp. CK23, CK24 and CK10, *Roseateles* sp. CK15, *Rhizobium* sp. CK19, *Burkholderia* sp. CK52, and unidentified strains, NI CK36, NI CK42, NI CK53, and NI CK54. The most efficient solubilization was indicated by the enlargement of the halo zones over time, despite the colony diameter staying relatively small (Figure 4).

Table 3. Bacteria with the highest solubilization of $Ca_3(PO_4)_2$ on NBRIP's medium at 4, 6 and 8 days after inoculation

Strain	4 days after inoculation			6 days after inoculation			8 days after inoculation		
	Halo zone dia (mm)	Colony dia (mm)	Solubilization Index	Halo zone dia (mm)	Colony dia (mm)	Solubilization Index	Halo zone dia (mm)	Colony dia (mm)	Solubilization Index
Erwinia sp. CK10	6.33	3.67	2.73bc	9.33	4.00	3.33b	10.7	4.00	3.67bc
Roseateles sp. CK15	8.67	2.00	5.33d	11.50	2.17	6.31cd	11.7	2.33	6.00cd
Rhizobium sp. CK19	10.3	3.67	3.82c	12.00	4.33	3.77bc	12.8	4.50	3.85bc
Enterobacter sp. CK23	9.33	2.00	5.67d	12.00	2.00	7.00d	12.5	2.50	6.00cd
NI CK36	3.83	2.67	2.44b	4.50	2.67	2.69b	7.33	3.00	3.44bc
NI CK42	4.17	2.17	2.92bc	4.17	2.17	2.92b	4.50	2.17	3.08b
Burkholderia sp. CK52	4.17	1.67	3.50bc	5.33	2.17	3.46b	6.67	2.67	3.50bc
NI CK53	6.67	1.67	5.00cd	10.33	1.83	6.64d	11.0	2.17	6.08cd
NI CK54	5.33	1.50	4.56cd	7.83	1.67	5.70cd	9.33	2.00	5.67cd

Notes: NI: unidentified microorganisms; Value is average of triplicate; SI values followed by the same letter are not significantly different at ($P < 0.001$) by Fisher's pairwise comparison; Strains unable to grow were excluded from screening.

In the secondary screenings, selected bacteria mineralized phytic acid more rapidly than that of the $Ca_3(PO_4)_2$ form of P (Table 4); by the third day after inoculation the mineralization zones started to merge among the colonies (Figure 4b). All identified and non-identified bacterial strains tested showed similar abilities for P solubilization activities in both media with pH 4.5 and 6.0 and reduced nitrogen source. Some bacteria could not grow in the secondary screenings plates probably due to modification of the pH (Table 4). However, none of these bacteria were able to grow on $AlPO_4$–containing medium, with or without $CaCl_2$ addition.

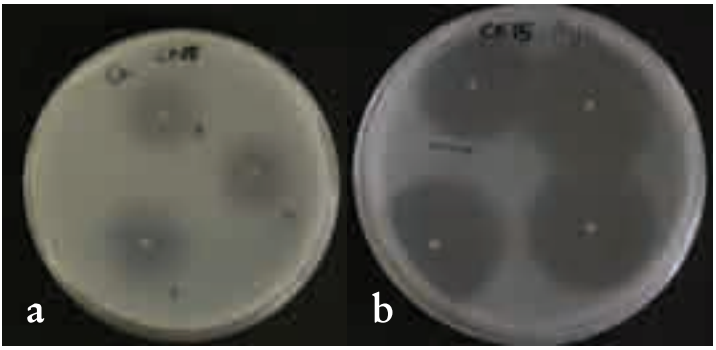


Figure 4. Phosphate solubilization by *Roseateles* sp. CK15 on modified NBRIP shown by clear zone (halo) around the colony. (a) $Ca_3(PO_4)_2$ pH 4.0, 4 days after inoculation, (b) phytic acid pH 4.0, 3 days after inoculation

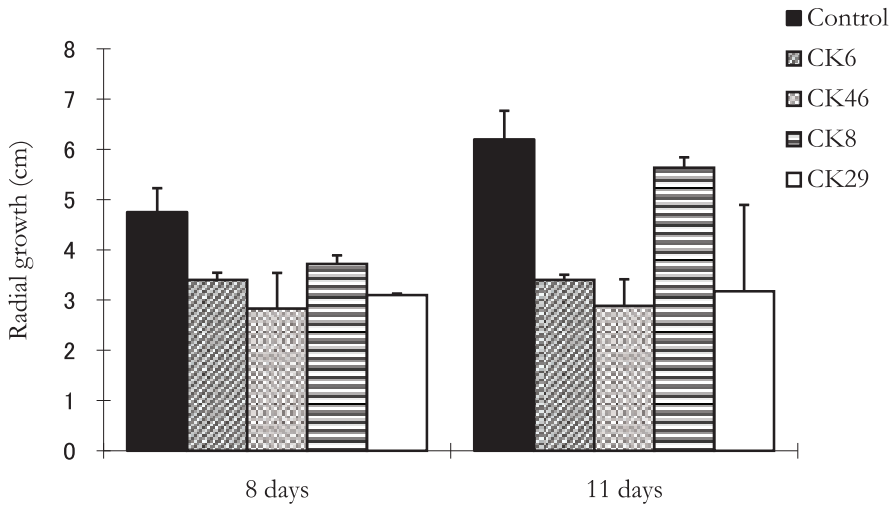


Figure 5. Quantitative radial growth of ectomycorrhizal fungus *Laccaria* sp. measured 8 and 11 days after co-inoculation. Bars indicated triplicates.

Table 4. Solubilization of phytic acid on NBRIP's media 3 days after inoculation

Strains	$C_6H_6(OPO_3H_2)_6$ (phytic acid) at pH 6 [1]			$Ca_3(PO_4)_2$ at pH 4.5 [2]		
	Halo zone dia (mm)	Colony dia (mm)	Solubilization index	Halo zone dia (mm)	Colony dia (mm)	Solubilization index
Erwinia sp. CK10	NG	NG	NG	9.67	3.33	3.90
Roseateles sp. CK15	30.38	2.13	15.29	11.00	3.00	4.67
Burkholderia sp. CK52	NG	NG	NG	NG	NG	NG
Rhizobium sp. CK19	32.13	2.88	12.17	12.67	3.33	4.80
Enterobacter sp. CK23	28.63	2.38	13.05	10.17	3.50	3.90
Erwinia sp. CK24	26.25	2.25	12.67	12.00	3.00	5.00
NICK26	NG	NG	NG	NG	NG	NG
NI CK42	NG	NG	NG	NG	NG	NG
NI CK53	28.88	2.38	13.16	NG	NG	NG
NI CK54	26.88	2.13	13.65	NG	NG	NG

Notes: NG: no growth on the medium; NI: unidentified microorganisms; Value is average of [1] 4 replicates and [2] 3 replicates; No statistically significant different of SI among bacteria at ($P < 0.05$) by Fisher's pairwise comparison

4. Nitrogen fixation activity

Four strains showed positive acetylene reduction activity in the initial screening: *Burkholderia* sp. CK32, *Azospirillum* sp. CK26, NI CK4, and NI CK55, thus they were subjected to further quantitative analysis for ethylene produced in triplicates (Figure 6a). Significant differences in the ethylene produced were observed at 6 and 9 days after acetylene injection ($P < 0.05$), in particular, *Azospirillum* sp. CK26 produced a higher ethylene than the control *B. indica* subsp. *indica* IFO 3744, followed by NI CK4, *Burkholderia* sp. CK32, and NI CK55. *Azospirillum* sp. CK26, *B. indica*, and NI CK4 increased their ethylene production at 6 days after acetylene injection. On the contrary, *Burkholderia* sp. CK32 and NI CK55 showed decreasing rates. At 9 days after acetylene injection, all except NI CK55 decreased the ethylene production. The amount of ethylene produced showed to have a positive correlation to the population density of the bacteria ($r = 0.71$ and 0.60, measured before and after ARA, respectively, Figure 6).

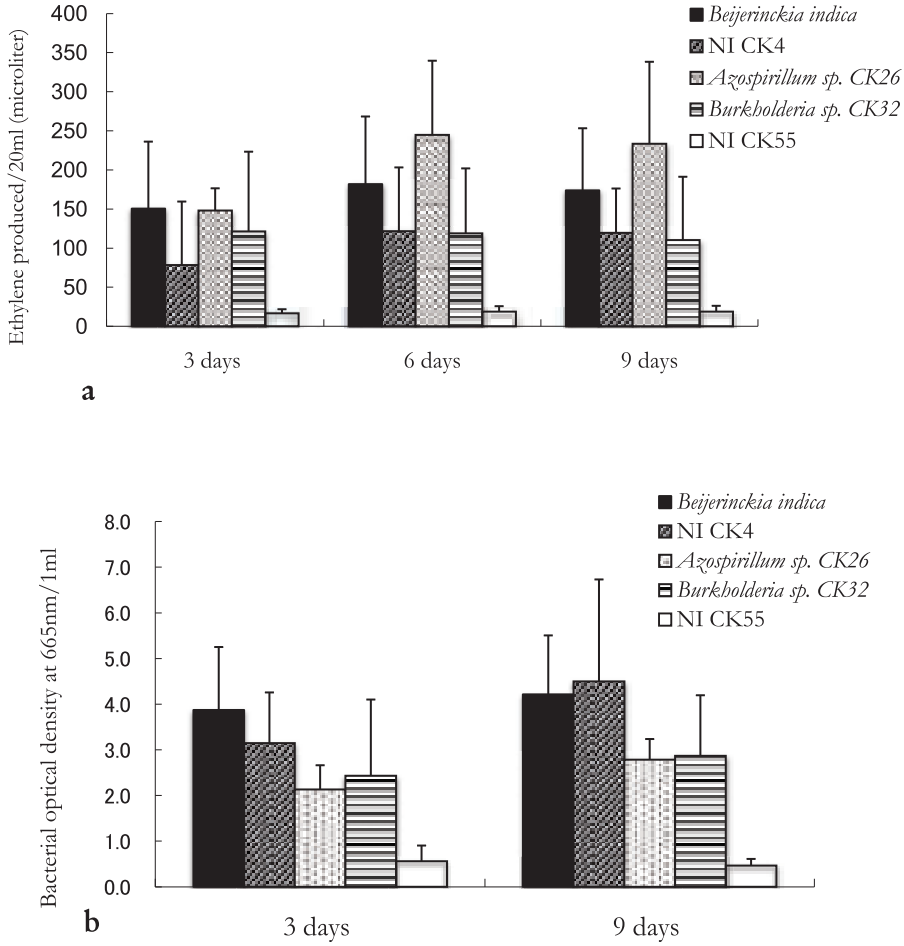


Figure 6. Ethylene (C_2H_4) production by four potential N_2 fixing bacteria. (a) Ethylene amount produced in 30-ml screw cap septum vials by nitrogen-fixing bacteria 3, 6 and 9 days after acetylene injection. Sixty seven strains with no positive acetylene reduction response in this medium were excluded. (b) Population of bacteria estimated by their optical density at 665 nm, 0 day before and 9 days after C_2H_2 injection. *Beijerinckia indica* subsp. *indica* IFO 3744 is the positive control of N_2 fixer. Bars indicated triplicate and statistically significant difference at $P < 0.05$ by Fisher's pairwise comparison.

5. Ectomycorrhization helper trait

Two steps of qualitative screenings were applied to observe the interaction between fungus *Laccaria* sp. and bacteria. Initial screening using an MW agar medium indicated that some bacteria promoted while others inhibited fungal mycelial growth. In particular, *Chromobacterium* sp. CK 8 promoted fungal growth but *Burkholderia* sp. CK46, *Frateuria* sp. CK 29, and *Klebsiella* sp. CK6 inhibited fungal growth with different levels of mycelial suppression.

To confirm this visual finding, selected bacteria were further analyzed microscopically and for this, two media, potato dextrose (PD) and MW agar plates were used. Growth of both ECM fungus and bacteria tested was faster on PD than on MW agar medium, and thus PD was used for subsequent test. Observation showed that *Chromobacterium* sp. CK8 accelerated the growth of *Laccaria* sp. of 3.7 cm, 8 days after co-inoculation and the fungus formed a thick, cord-like structure as they were approaching the bacterial colony (Figure 8d). The blank, solely consisted of *Laccaria* sp, with the radial growth of 4.8 cm, also had a similar hyphal structure but a less thick cord structure than of that formed in the presence of *Chromobacterium* sp. CK8 (Figure 8a). Inoculation with *Burkholderia* sp. CK46 resulted in the shortest radial growth of the fungus of 2.83 cm. The hyphae showed a slimmer and sharper structure than the blank, and they profusely formed a bundle-like structure as they approached the bacterial colony (Figure 8c). Co-inoculated with *Klebsiella* sp. CK6, the hyphae also formed a bundle-like structure as they approached the bacterial colony (Figure 7a and 7b) although they approached the bacterial colony faster than that of *Burkholderia* sp. CK46. Further, in the presence of *Frateuria* sp. CK29, the fungus grew profusely towards the bacteria although they had shorter hyphae than the blank and took a long time to reach the bacterial colony (Figure 8e). Two isolates, *Chromobacterium* sp. CK8 and *Frateuria* sp. CK29, were fully colonized by fungal mycelia after one month of incubation. The fungus *Laccaria* grew well but slower than fungus grown with *Chromobacterium* sp. CK8. For dual assay between *Laccaria* sp. and *Klebsiella* sp. CK6 or *Burkholderia* sp. CK46, we observed a clear interfering zone in between mycelia and these bacterial colonies on the plates until the end of the observation (2 months), although microscopic observation showed that some mycelia were eventually able to grow inside the bacterial colony (Figure 9).

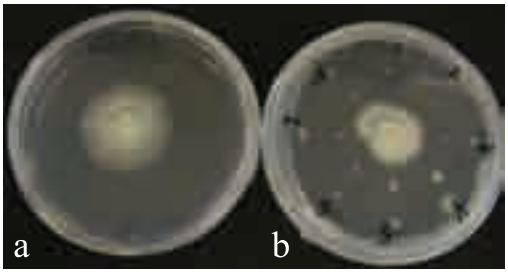


Figure 7. Initial screening of ectomycorrhization bacteria on MW agar plate. (a) *Laccaria* sp. only, as blank, and, (b) *Laccaria* sp. and eight strains of bacteria inoculated twice around the mycelial colony

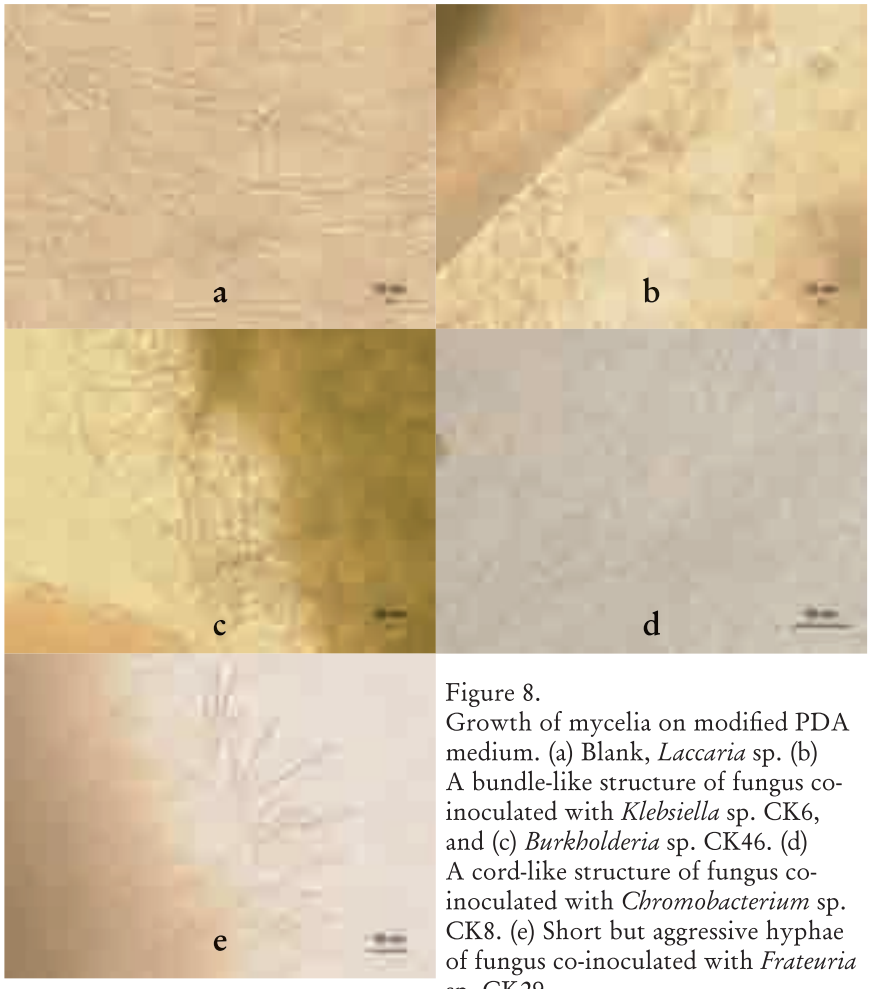


Figure 8.
Growth of mycelia on modified PDA medium. (a) Blank, *Laccaria* sp. (b) A bundle-like structure of fungus co-inoculated with *Klebsiella* sp. CK6, and (c) *Burkholderia* sp. CK46. (d) A cord-like structure of fungus co-inoculated with *Chromobacterium* sp. CK8. (e) Short but aggressive hyphae of fungus co-inoculated with *Frateuria* sp. CK29

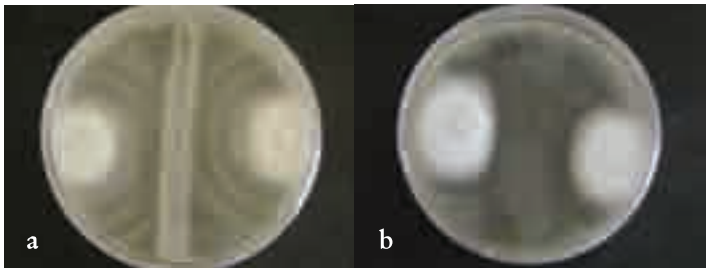


Figure 9. Interaction of ectomicorrhizal fungi and bacteria on MW agar. (a) mycelial growth promotion by bacteria; (b) a clear interfering zone in between mycelia and these bacterial colonies.

Further, quantitative analysis showed that 11 days after co-inoculation, only *Laccaria* sp. co-inoculated with *Chromobacterium* sp. CK8 had radial growth of 5.6 cm which was almost as wide as that of the blank of 6.2 cm. *Burkholderia* sp. CK46 and *Frateuria* sp. CK29 had the shortest radial growth of 2.8 and 3.2 cm, respectively at 11 days after co-inoculation.

B. Discussion

For the purpose of this study, nitrogen-free medium was efficient for trapping oligotrophs, especially because the nitrogen available for plants is limited in peat. This medium enabled isolation of diverse, associative bacterial communities of predominantly Gram-negative bacteria belonging to α-, β-, or γ-subclasses of Proteobacteria from dipterocarps in Central Kalimantan peat swamp lands. The genus *Burkholderia* is the predominant culturable colonizers in the rhizosphere and the rhizoplane. *Burkholderia* spp. have been reported to be common composers of soil microfloral communities (Salles *et al.*, 2002), and many of their species are PGPR of many plant species. Nitrogen-fixing *Burkholderia* spp. were isolated from rhizosphere, rhizoplane, and inner tissues of sugarcane, maize, and teosinte plants in Brazil, Mexico, and South Africa (Reis *et al.*, 2004). A P-solubilizing bacterium *Burkholderia cepacia* CC-Al74 was isolated from soil in Pingdong, Taiwan (Lin *et al.*, 2006). Two of the most potent PGPR *in vitro* in our study belonged to *Burkholderia* spp.: CK52 isolated from *Hopea* sp., and CK32 isolated from *S. belangeran*, as they had high P solubilization and nitrogen fixation activities, respectively. In contrast, *Rhizobium* spp. CK48, CK19 and CK27 did not show any nitrogen fixing ability as indicated by ARA, although these genera are known as nitrogen-fixing bacteria. In a free-living stage, *Rhizobium leguminosarum* biovar. *phaseoli* has been reported to have P solubilization activities (Chabot *et al.*, 1996; Rodriguez and Fraga, 1999) rather than fix N. However, in the screenings for P solubilizers and N_2 fixers, none of our bacteria showed bifunctional characteristics.

Roseateles sp. CK15, *Rhizobium* sp. CK19, *Enterobacter* sp. CK23, and *Erwinia* sp. CK24 solubilized/mineralized both of the P sources suggested that peat swamp ecosystem in Central Kalimantan is rich in bacteria that effectively utilize unavailable P for host plants. The P solubilization mechanism may involve solubilization of inorganic P by organic acid that is bio-synthesized by soil microorganisms. Gluconic acid has been reported as the major factor for the high rate of $Ca_3(PO_4)_2$ solubilization by *Burkholderia cepacia* CC A174 (Lin *et al.*, 2006). It was suggested that the acidification and chelation by gluconic acid and 2-keto-gluconic acid lead to the dissolving of $Ca_3(PO_4)_2$. Another mechanism is: mineralization of organic P in peat soil increases in the rhizosphere by means of acid phosphatase and/or phytase (Tarafdar and Junk, 1987, Rodriguez and Fraga, 1999). Richardson and Hadobas (1997) reported that 63% of culturable soil bacteria were able to grow on agar medium with phytate as sole carbon and P sources. In the case of the dipterocarp-associating bacteria, *in vitro* mineralization of phytic acid occurred more rapidly than the inorganic $Ca_3(PO_4)_2$, indicating that the bacteria probably

play an important role in P uptake in the ecosystem. Further study on the ability of these potent P solubilizers for root P-uptake *in situ* will be our future approach.

Dipterocarp is an ectomycorrhiza-dependent tree, therefore an assay to investigate the interaction of these bacteria with the test fungus *in vitro* would provide a preliminary understanding on the mechanism of ECMHB. Using *Laccaria* sp. as a test ectomycorrhizal fungus, the assay suggested two distinct phenomena. Growth promotion was indicated by the formation of a cord-like structure as the fungi approached the bacterial colony, and inhibition was indicated by a slim, sharp and bundled-like structure around the bacterial colony. The most distinct growth-promoting activity was shown by *Chromobacterium* sp. CK8 and this is the first time that the genus *Chromobacterium* is reported to have promoting activity towards ectomycorrhizal fungus *Laccaria* sp. *in vitro*. A study reported that *Chromobacterium violaceum* was associated with *Glomus intraradices,* a VAM fungus (Mansfeld-Giese *et al*., 2002). *Frateuria* sp. CK29 in particular, inhibited the radial growth of fungus in the quantitative assay, 11 days after co-inoculation although the bacteria were eventually overlapped by the fungus at the end of the observation. However, plate is a limited space with a limited nutrient available for both organisms to have optimum growth. It is thus suggested that *Chromobacterium* sp. is a true ECMHB *in vitro* because of the ability to promote the radial growth of fungus almost as wide as that of the blank while at the same time the bacterium had to compete with a limited nutrient. On the contrary, *Burkholderia* sp. CK46 inhibited the hyphal growth from coming near the bacterium colony as shown by the accumulation of bundled hyphae at the interfering zone. It is thus speculated that the bacterium exudes antagonistic substrate to the surrounding plate and inhibits the growth of mycelia approaching the bacterium.

This research provides preliminary information on diverse bacteria associated with dipterocarps and their potent PGPR properties *in vitro*. This is probably the first study to reveal that mixed dipterocarp forests in Central Kalimantan are a good source for the isolation of diverse genera of associative functional rhizobacteria showing potential as PGPR *in vitro*. The traits were not exclusive to specific genera, stressing the importance of an *in vitro* screening of a wide range of genera to characterize their potential PGPR traits. These results, however, are yet to be confirmed by an inoculation to their host seedlings to reveal their actual PGPR properties. Our future approach will be directed towards investigating the effects of these potent PGPR traits on plants under nursery and field conditions. The use of mixed inoculants to obtain a more balanced nutrient acquisition by plants will also be targeted. It is typical that transplanted seedlings grown in degraded areas having acidic soil, toxic elements, and low nutrient content suffer low survival rates. Therefore, PGPR can offer a biological source of nutrient for supporting growth of saplings in these degraded lands.

IV. CONCLUSION

Diverse bacteria were associated with rhizosphere and rhizoplane of mixed dipterocarp forests of acidic peat land in Central Kalimantan. The forests were a good source for the isolation of diverse genera of associative functional rhizobacteria showing potential as PGPR *in vitro.* Fourteen strains of diverse genera from the total 71 possessed PGPR traits of P solubilization, N fixation, and ectomycorrhization *in vitro*. Further studies will involve inoculation of PGPR onto seedlings and transplantation of the saplings on degraded lands in order to see the effect of PGPR on growth of the host.

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