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Biodiversity of plant growth promoting rhizobacteria from rhizosphere of *Pinus* sp and *Casuarina* sp at Raden Soerjo Forest Park

Brigita Nur Diyan Agustiana[1], Indra Gumilar[1], RizqiaNurul Alfiyanita[1], Alifah Nastiti[1], Tesalonika Tetya Virginia[1], Siti Rizqiyatul Mukarromah[1], Annisa Dwi Savitri[1], Lunace Rahma Kinayoh[1], Erna Erviana Nadhifa[1], Khafid Jallaludhin[1], Ni'matuzahroh[12] and Almando Geraldi[12*]

[1]Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia
[2]Research Center for Bio-Molecule Engineering, Universitas Airlangga, Surabaya, Indonesia

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ABSTRACT

Indonesia is one of the megadiversity countries in the world endowed with rich and unique biodiversity of microorganisms, including plant growth promoting rhizobacteria (PGPR). PGPR is considered as more sustainable, economical, and environmentally friendly alternatives to chemical fertilizers in various industries including pine plantation industries. This research aims to explore the biodiversity of PGPR in the rhizosphere of *Pinus* sp and *Casuarina* sp at Raden Soerjo Forest Park, East Java, Indonesia. Researchers successfully isolated a number of morphologically distinct microbial colonies; nitrogen fixing-, phosphate solubilizing, cellulose degrading-, and plant hormone producing- bacteria from the rhizosphere of *Pinus* sp and cellulose degrading, and plant hormone producing- bacteria from the rhizosphere of *Casuarina* sp. This research is one of the initial efforts to utilize native PGPR from Indonesia to increase the yield of farming, plantation, and other related industries in Indonesia.

Key words: *Rhizobacteria, Plant growth promoting bacteria, Pinus* sp., *Casuarina* sp., *Biofertilizer*

Introduction

Rhizosphere known as a region around plant's root. There has diverse number of microorganisms which living in for instance plant growth-promoting rhizobacteria (PGPR). Plant growth-promoting rhizobacteria (PGPR) stimulate the growth of their host plants. On the basis of their relationship with the plants, PGPR have been divided into two major groups: symbiotic and free-living (Khan, 2005; Hayat *et al*. 2010). PGPR have three main features: (i) root colonization ability, (ii) high survivability and multiplicity in root surroundings helping in plant growth promotion, and (iii) inhibition of phytopathogens (Lugtenberg *et al*., 2001; Gamalero *et al*., 2004). Different plants and environment having specific dominant PGPR species. Root microorganisms of plants depend on various environmental (biotic and abiotic) factors such as root type, plant species, plant age, soil type (Campbell, 1985) and type of plant species (genotypes) (Lareen *et al*., 2016).

PGPR is considered as more sustainable, economical and environmentally friendly alternatives to chemical fertilizers, beneficial for lower produc-

tion cost as well as recognize the best soil and crop management practices to achieve more sustainable agriculture as well as fertility of soil (Maheshwari *et al.*, 2012). PGPR as biofertilizer has been proven as a safe and efficient method of increasing crop yields (Premachandra *et al*., 2016; Vejan *et al*., 2016). In recent years considerable attention has been paid to PGPR to replace agrochemicals (fertilizers and pesticides) for the plant growth promotion by a variety of mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, crucial for soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation (Sivasakhti *et al.,* 2014). Recently from last few decades numerous bacterial genera such as *Azotobacter, Bacillus, Klebsiella, Enterobacter, Arthrobacter, Burkholderia, Pseudomonas, Serratia*, etc. had been used as biofertilizers as reported by various authors and called these isolates as PGPR. (Kloepper *et al*., 2004; Saharan and Nehra, 2011; Kumar *et al*., 2014, 2015a, 2016a,b, 2017a,b; Singh *et al*., 2017a).

Materials and Methods

Sampling site and methods

Sampling was doing by indirect method which mean sample was taken based on the course of the research and the situation at the site. Soil samples were collected from five sampling points at each vegetation in rhizosphere of *Pinus* sp and *Casuarina* sp. Temperature, soil humidity, pH of soil, light intensity, and coordinate were measured before collecting the sample using silinder crop. Samples were then stored in a sterile container for a day until next treatment at the laboratory.

Isolation of bacteria

Soil samples were take from five points in every sampling site was mixed and homogenized. Five g of soil sample was diluted in 45 mL of sterile NaCl 0.85% in 100 mL conical flask and kept in shaker to get a homogenized soil suspention (Geraldi *et al.*, 2019). Serial dilution which isolate was 10^{-8}, 10^{-9}, and 10^{-10}. For every soil sample were spread onto TSA (Tryptic Soy Agar) for a plant hormone producing bacteria such as IAA (A'ini, 2013), CMC (Carboxy Methil Cellulose) for the cellulose degrading bacteria which has a cellulolytic activity (Geraldi *et al.*, 2019), NFA (Nitrogen Fixation Agar) for nitrogen fixing bacteria and Pikovskaya for phosphate solubilizing bacteria (Maristha *et al*, 2013). And then incubated at 30 °C (Geraldi *et al.*, 2019) for 216 hours.

Identification of bacteria

Identification of bacteria was by macroscopic and microscopic characterized. Size, shape, color, consistency, margin and elevation were the macroscopis characterized. The microscopic characterized was Gram staining, it was able to know the type Gram of the bacteria. First took the colonies and pinned on the object glass. Secondly, drop a few of cristal violet as a primary color for a minute then washed using aquades. Flushing lugol as mordant for a minute and washed using aquades. Then, give alcohol as the decolorisator for 30 seconds. Last, use safranin as a competitor color for 30 seconds and wash using aquades.

Screening of Cellulolytic Activity of bacteria "P"

Single colony of the bacteria "P" was streak onto CMC agar plate and incubated at 30 °C for 48 hours. After incubation, the CMC agar was overflown with Congo Red solution (1% w/v) for 30 minutes. Then, congo red was discarded and the plates were futher treated by flooding with 1 M NaCl for 30 minutes. Cellulolytic activity confirmed by clear zone surrounding colonies (Geraldi *et al*., 2019).

Screening of biosurfactants production of bacteria"P":

The ability of bacteria "P" to produce biosurfactants was screened using hemolytic activity assay (Ibrahim, 2018). In this assay, bacteria "P" was streak onto NA (Nutrien Agar) that was supplemented with 5% sheep blood and then incubated at 40 °C for 48 hours. The formation of hemolysis result and clear zone color indicated hemolytic type (Geraldi *et al*, 2019).

Sreening of Lypase production of bacteria "P"

The ability of bacteria "P" to produce lypase was screened using rhodamine medium, lypase production confirmed by orange fluorescent under ultraviolet.

Results and Discussion

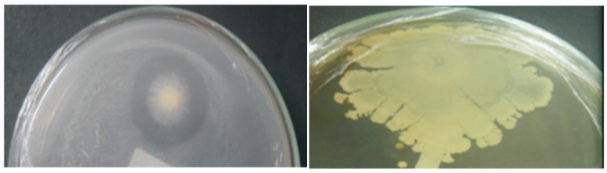
Research about Plant Growth Promoting Rhizobacteria at Raden Soerjo Forest Park done in rhizhosphere of *Pinus* sp in coordinate 07°44'21,7" S Latitude 112°32'00,6" E Longitude at 5380 mdpl and rhizosphere of *Casuarina* sp in coordinate 0744'26,0" S Latitude 112°32'05,9" E Longitude at 5327 mdpl in summer season. Each rhizosphere has it is group of plant growth promoting rhizobacteria such as phosphate solubilizing-, nitrogen fixing-, cellulose degrading-, and plant hormone producing. Isolation started by doing in a selective medium and identification. The result of isolation in a selective medium showing a different morphological microbial colonies such as form, pigmentation, elevation, consistency, size, and margin. Microscopic identification showing form and Gram staining of the colonies.

Table 1. Environmental parameters of rhizosphere of *Casuarina* sp and *Pinus* sp at Raden Soerjo Forest Park

Rhizosphere	*Casuarina* sp	*Pinus* sp
Humidity	80°RH	90.5°RH
Soil Moisture	52.92%	45.31%
pH of Soil	6.4	6.3
Light Intensity	≥3000 Cd	≥3000 Cd

The data show that most of the colonies form were circular and has small size while the pigmentation were yellowish white transparently with the flat margin. Gram staining of the colonies show the positive Gram were dominant and the microscopic form were coccus.

We also did doing cellulase, lypase, and hemolysis screening for cellulose degrading bacteria. The result of the screening show that code of "P" bacteria has a activity for hydrolysis cellulase which confirmed by halozone around colonies, lipase activity positive confirmed by orange fluorescent showing by colonies under ultraviolet, and biosurfactant which confirmed by clear zone surrounding colonies.



a. b.

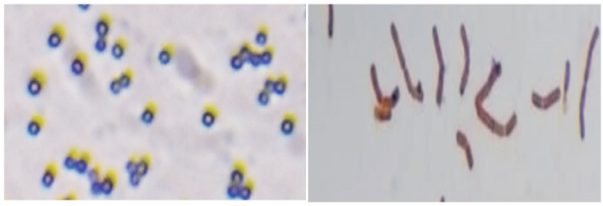
Fig. 1. Morphological macroscopic colonies at (a.) Pikovskaya medium and (b.) TSA medium isolate at 37°C

Table 2. Macroscopic Characteristic of Plant Growth Promoting Rhizobacteria from Rhizosphere of *Pinus* sp and *Casuarina* sp

No.	Code of bacteria	Size	Pigmentation	Form	Margin	Elevation	Consistency
1.	a	Large	Yellowish white	Irregular	Undulate	Flat	Transparent
2.	b	Large	Yellowish white	Irregular	Rhizoid	Flat	Transparent
3.	c	Small	Yellowish white	Circular	Entire	Umbonate	Opaque
4.	d	Small	Yellowish white	Circular	Serrate	Flat	Transparent
5.	e	Large	Yellowish white	Irregular	Lobate	Flat	Opaque
6.	f	Pinpoint	White	Circular	Irregular	Flat	Transparent
7.	g	Small	White	Circular	Irregular	Flat	Transparent
No.	Code of Bacteria	Size	Pigmentation	Form	Margin	Elevation	Consistency
8.	h	Moderate	Yellowish white	Irregular	Rhizoid	Flat	Transparent
9.	i	Small	Yellowish white	Circular	Entire	Flat	Opaque
10.	j	Small	White	Irregular	Serrate	Flat	Transparent
11.	k	Small	Yellowish white	Circular	Entire	Flat	transparent
12.	l	Small	Yellowish white	Irregular	Irregular	Flat	Transparent
13.	m	Small	Yellowish white	Circular	Lobate	Flat	Transparent
14.	n	Small	Yellowish white	Circular	Undulate	Flat	Transparent
15.	o	Small	Yellowish white	Circular	Entire	Raised	Opaque
16.	p	Small	White	Circular	Entire	Flat	Transparent
17.	q	Small	Yellow	Circular	Entire	Flat	Opaque
18.	r	Small	White	Circular	Filamen	Flat	Opaque
19.	s	Small	Yellow	Irregular	Serrate	Raised	Opaque

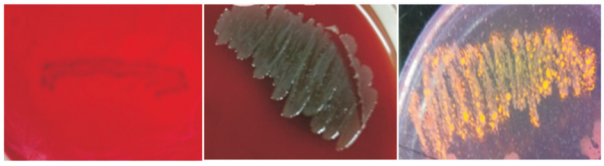
Table 3. Microscopic Characteristic and Gram Staining of Plant Growth Promoting Rhizobacteria from *Pinus* sp and *Casuarina* sp

No.	Rhizhosphere	Selective Medium	Code of Bacteria	Gram Staining	Microscopic Form
1.	*Casuarina* cp	TSA	a	+	Coccus
2.			b	+	Coccus
3.			c	+	Coccus
4.			d	+	Coccus
5.			e	+	Cocoid
6.		CMC	f	+	Coccus
7.			g	+	Coccus
8.	*Pinus* sp	TSA	h	+	streptobacil
9.			i	-	Streptobacil
10.			j	-	Streptobacil
11.			k	+	Coccus
12.			l	-	Streptobacil
13.			m	+	Coccus
14.			n	+	Coccoid
15.			o	+	Coccoid
16.		CMC	p	+	Coccus
17.		NFA	q	+	Coccus
18.		Pikovskaya	r	+	Coccus
19.			s	+	Coccus



a. b.

Fig. 2. Morphological microscopic colonies of (a.) "m" Gram positive coccus and (b.) "J" Gram negative streptobacil.



a. b. c.

Fig. 3. Cellulose activity of (a.) "p" in CMC medium overflow by congo red confirmed by halozone surrounding colonies and (b.) hemolysis activity of "p" confirmed by clear zone around colonies, while (c.) colonies "p" in a medium rhodamine confirmed by orange fluorescent under ultraviolet.

Conclusion

Biodiversity of plant growth promoting rhizobacteria from rhizosphere of *Pinus* sp contain bacteria of phosphate solubilizing-, nitrogen fixing -, cellulose degrading-, and plant hormone producing-, while from rhizosphere of *Casuarina* sp contain bacteria of cellulose degrading-, and plant hormone producing-. Based on the result of this research, research about biodiversity of plant growth promoting rhizobacteria from rhizosphere of *Pinus* sp and *Casuarina* sp well-earned to know the species of the bacteria. Application of plant growth promoting rhizobacteria in Indonesia will give an efforts to increase the yield of farming, plantation, and other related industries. The utilization of Plant Growth Promoting Rhizobacteria from this research will give a benefit for our society through more researches. The result of these research also give reference for other researches related to plant growth promoting rhizobacteria

References

A'ini, Zakiyah Fitrah, 2013. Isolasidan Identifikasi Bakteri Penghasil IAA (Indole-3-Acetid Acid) dari Tanah dan Air di Situgunung, Sukabumi. *Faktor Exacta.* 6(3): 231-240.

Campbell, R. 1985. *Plant Microbiology* (p. 191). Baltimore: Edward Amold.

Das, N.P., Kumar, A. and Singh, P.K. 2015. Cyanobacteria, Pesticides and Rice Interaction. *Biodivers Conserv.* 24 (4): 9951005. http://doi.org/10.1007/s10531-015-0886-8

Dash, N. P., Kumar, A., Kaushik, M. S., Abraham, G.,

Singh, P. K., Kaushik, M.S. and Abraham, G. 2017a. Agrochemicals Influencing Nitrogenase, Biomass of N2-Fixing Cyanobacteria and Yield of Rice in Wetland Cultivation. *Biocatal Agric Biotechnol*. 9 : 2834. http://doi.org/10.1016/j.bcab.2016.11.001

Dash, N. P., Kumar, A., Kaushik, M. S., Abraham, G. and Singh, P. K. 2017b. Nitrogenous Agrochemicals Inhibiting Native Diazotrophic Cyanobacterial Contribution in Wetland Rice Ecosystem. *J Appl Phycol.* 29 (2) : 929939. http://doi.org/10.1007/s10811-016-0998-x

Gamalero, E., Lingua, G., Caprì, F. G., Fusconi, A., Berta, G. and Lemanceau, P. 2004. Colonization Pattern of Primary Tomato Roots by *Pseudomonas fluorescens* A6RI Characterized by Dilution Plating, Flow Cytometry, Fluorescence, Confocal and Scanning Electron Microscopy. *FEMS Microbiology Ecology*. 48: 79–87. http://doi.org/10.1016/j.femsec. 2003.12.012

Geraldi, A., Ni'matuzahroh., Aken, P.W., Sucipto, H., Brigita, N.D.A., Alifah, N., Rizqia, N.A., Indra, G., Tesalonika, T.V., Siti, R.M., Annisa, D.S., Erna, E.N., Khafid, J. and Lunace, R.K. 2019. Bioprospecting of Cellulolytic and Biosurfactant-Producing Bacteria for Organic Waste Treatment. EM International. *Poll Res.* 38 : S114 – S117. https://www.researchgate.net/publication/336252112_ Biopropecting_of_Cellulolytic_and_Biosurfactant-_Producing_Bacteria_for_Organic_Waste_Treatment

Hayat, Q., Hayat, S., Irfan, M. and Ahmad, A. 2010. Effect of Exogenous Salicylic Acid Under Changing Environment: A review. *Environmental and Experimental Botany.* 68 : 14–25.http://dx.doi.org/10.3382/ps.2009-00575

Ibrahim, H. M. M. 2018. Characterization of Biosurfactants Produced by Novel Strain of *Ochrobactrum anthropic* HM-1 and *Citrobacter freundii* HM-2 from Used Engine Oil-Contaminated Soil. *Egyptian Journal of Petroleum*. 27(1) : 21-29. https://doi.org/10.1016/j.ejpe.2016.12.005

Khan, A. G. 2005. Role of Soil Microbes in the Rhizosphere of Plants Growing on Trace Metal Contaminated Soils in Phytoremediation. *Journal of Trace Elements in Medicine and Biology.* 18 : 355-364.https://doi.org/10.1016/j.jtemb.2005.02.006

Kloepper, J.W., Lifshitz, R. and Zablotowicz, R.M. 1989. Free-Living Bacterial Inocula for Enhancing Crop Productivity. *Trends Biotechnol.* 7 : 3943.https://doi.org/10.1016/0167-7799(89)90057-7

Kloepper, J. W., Ryu, C. M., Zhang, S., Ryu, C.M. and Zhang, S.A. 2004. Induced Systemic Resistance and Promotion of Plant Growth by Bacillus spp. *Phytopathology.* 94 : 1259-1266. https:/apsjournals.apsnet.org/doi/abs/10.1094/PHYTO.2004.94.11.1259

Kumar, A., Singh, R., Giri, D.D., Singh, P.K. and Pandey, K.D. 2014. Effect of *Azotobacterchrococcum* CL13 Inoculation on Growth and Curcumin Content of Turmeric (*Curcuma longa* L.). *Int J Curr Microbiol Appl Sci.* 3 (9), 275283.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5624808/

Roy, B.K. 2015a. Isolation and Characterization of Bacterial Endophytes from The Roots of *Cassia tora* L. *Ann Microbiol.* 65 : 1391 1399.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4752947/

Kumar, A., Singh, R., Yadav, A., Giri, D. D., Singh, P. K., Pandey, K. D. and Yadav, A. 2016a. Isolation and Characterization of Bacterial Endophytes of *Curcuma longa* L. 3. *Biotech*. 6 : 60. http://doi.org/10.007/s13205-016-0393-y

Lareen, A., Burton, F. and Schäfer, P. 2016. Plant Root-Microbe Communication in Shaping Root Microbiomes. *Plant Molecular Biology*. 90 : 575–587.https://doi.org/10.1007/s11103-015-0417-8

Lugtenberg, B. J. J., Dekkers, L. and Bloemberg, G. V. 2001. Molecular Determinants of Rhizosphere Colonization by Pseudomonas. *Annual Review of Phytopathology.* 39: 461–490.https://doi.org/10.1146/annurev.phyto.39.1.461

Maristha, Etha, Siti Khotimah and Riza Linda, 2013. Bakteri Pelarut Fosfat Hasil Isolasidari Tiga Jenis Tanah Rhizosfer Tanaman Pisang Nipah (*Musa paradisiaca* Var. nipah) di Kota Singkawang. *Protobiont.* 2 (2) : 93-101. Pontianak.

Maheshwari, D.K., Dubey, R.C., Aeron, A. and Kumar, B. Kumar S. 2012. Integrated Approach for Disease Management and Growth Enhancement of *Sesamum indicum* L. Utilizing *Azotobacter chroococcum* TRA2 and Chemical Fertilizer. *World J Microbiol Biotechnol.* 28 : 3015-3024. http://doi.org/10.1007/s11274-012-1112-4

Premachandra, D., D., Hudek, L. and Brau, L. 2016. Bacterial Modes of Action for Enhancing of Plant Growth. *J Biotechnol Biomater.* 6 : 326.https://doi.org/10.4172/2155-952x.1000236

Saharan, B.S. and Nehra, V. 2011. Plant Growth Promoting Rhizobacteria: A Critical Review. *Life Sci Med Res.*/ 21 (1) : 30.http://astonjournals.com/manuscripts/Vol2011/LSMR-21_Vol2011.pdf

Sivasakhti, S., Usharani G. and Saranraj P. 2014. Biocontrol Potentiality of Plant Growth Promoting Bacteria (PGPR) – Pseudomonas fluorescence and Bacillus subtilis : A Review. *African Journal of Agricultural Research.* 9: 1265-1277. https://doi.org/10.5897/AJAR2013.7914

Vejan, P., Rosazlin, A., Tumirah, K., Salmah, L. and Amru, N.B. 201. Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability-a review. *Molecules.* 21: 573. http://doi.org/10.3390/molecules21050573

Vessey, J. 2003. Plant Growth Promoting Rhizobacteria as Biofertilizers. *Plant and Soil.* 255 : 571-586. https://www.jstor.org/stable/24123974