

## Diversity, activity, and effectiveness of *Rhizobium* bacteria as plant growth promoting rhizobacteria (PGPR) isolated from Dieng, central Java

Sri Purwaningsih\*, Dwi Agustiyani, Satjiya Antonius

Department of Microbiology, Research Center for Biology-Indonesian Institute of Sciences, Jalan Raya Cibinong, Cibinong, West Java, Indonesia

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### ABSTRACT

**Background and Objectives:** This study was aimed to isolate *Rhizobium* spp., from the plant rhizosphere and to investigate their effects on the growth of peanut (*Arachis hypogaea* L.) as plant growth-promoting rhizobacteria (PGPR).

**Materials and Methods:** The isolates were characterized using YEMA, YEMA + Congo Red, and YEMA + Bromothymol blue (BTB) media. The *Rhizobium* was tested qualitatively for their ability to produce indole acetic acid (IAA), siderophores, proteases, nitrogenases as well as phosphate solubilizing activity. A greenhouse experiment was carried out to elucidate the effect of *Rhizobium* inoculation on *Arachis hypogaea* L. growth.

**Results:** Eleven isolates were obtained in YEMA media and they were red-pink in the YEMA + Congo Red media. The YEMA + BTB test showed that 2 isolates were slow-growing and the rest were fast-growing isolates. Seven isolates produced siderophores, 5 were capable of phosphate solubilizing, 9 isolates produced protease enzyme, 4 isolates could produce IAA, and 7 isolates could fix nitrogen. The B1 and the combination of some high trait-isolate treatments in Y gave the best results on *Arachis hypogaea* L. growth.

**Conclusion:** These isolates can be developed as biological fertilizer agents for the peanut plant.

**Keywords:** Arachis; Fertilizers; Greenhouse; *Rhizobium*; Yeast extract mannitol agar

### INTRODUCTION

Dieng is a hilly highland region in Central Java, Indonesia that produces various vegetables, such as peanuts (*Arachis hypogaea* L.). Continuous peanut planting up to the top of the hills brings yield losses compared to planting other types of crops. Besides

the substantial production costs, pesticides and fertilizer usage are also continuous and excessive, which has caused chemical contamination. Thus, efforts need to be taken to improve the level of soil fertility. Among other ways, planting crops and using microorganisms to improve soil fertility without damaging the environment while still increasing the plants' productivity is the preferred method (1).

There are a lot of soil microbes that could fertilize the soil and increase plant's yields, one of them is *Rhizobium*. These bacteria are capable to live in the soil or inside the legume root nodules and fix nitrogen ( $N_2$ ) from the atmosphere to supply N to plants (2). Biological nitrogen fixation is estimated to contribute more than 170 million tons of nitrogen

\*Corresponding author: Sri Purwaningsih, Ph.D, Department of Microbiology, Research Center for Biology-Indonesian Institute of Science, Jalan Raya Cibinong, Cibinong, West Java, Indonesia.

Tel: +62-21-87907604

Fax: +62-21-87907612

Email: sipur2005@yahoo.co.id

to the biosphere annually, 80% of which is the result of *Rhizobium* bacteria and leguminous plants symbiosis (3).

Each *Rhizobium* species or strain has different abilities in symbiosis. A *Rhizobium* strain capable to form root nodules and nitrogen fixation is called a useful strain. Since not all *Rhizobium* species are capable to form root nodules with every legume, there is a need to isolate indigenous species and characterize them into cross-inoculation groups with the potential of developing a symbiotic relationship with specific legumes (4).

*Rhizobium* inoculation can meet the nitrogen needs in plants and reduce the need for inorganic nitrogen fertilizer (5). Effective inoculation with *Rhizobium* can provide up to 50-70% of the total nitrogen needs to increase yields (6). Besides fixing nitrogen, *Rhizobium* produces vitamins, amino acids, siderophores, and auxins (7). Besides *Rhizobium* is capable to produce indole acetic acid (IAA) and gibberellic acid (GA), which are growth hormones for germination and plant growth (8) and enhancing the growth of root hair and root branches that extend the root's range. *Rhizobium* can increase the absorption of phosphate (9). Phosphate is a primary nutrient in root development and the formation of soybean pods (10). The application of rhizobium inoculum in peanut plants can increase the root nodules, which function to fix nitrogen for plants. Nitrogen has an important role in the growth of leaves, stems, roots, flowers, and fruit. Besides, inoculation is expected to improve soil structure, balance soil pores, and increase soil resistance (11).

The effectiveness of *Rhizobium* in plants is influenced by several factors, such as the competition with native microbial populations and biotic agents like diseases and insects (12); the lack of limiting factors in the soil, such as phosphorus and various microelements (13); and low soil pH (below 5.0). Another problem is the diverse efficiency of *Rhizobium* inoculants for certain types of leguminous crops (14).

Thus, this research was aimed to understand the activity of the plant growth-promoting rhizobacteria (PGPR) *Rhizobium* spp. from the plant rhizosphere and soil, obtain pure cultures, and study their effects on the growth of peanut (*Arachis hypogaea* L.). In the end, this study is aimed to obtain potential isolates that can be developed as biological organic fertilizer agents. This study would help in the exploration of native rhizobium strains in promoting peanut

yield and improving soil fertility.

## MATERIALS AND METHODS

**Bacterial isolation and purification.** The composite soil samples were taken randomly at the depth of 0-15 cm from Dieng, Wonosobo, Central Java (Table 1). The samples were taken in March 2015. The bacterial isolation was prepared by diluting a 1 ml solution of soil and root sample into 9 ml of 0.85% NaCl until a  $10^{-1}$  to  $10^{-5}$  dilution series. About 0.1 ml of the final diluted solution was poured into a petri dish containing YEMA media and incubated at 27-28°C for 2-5 days. The colonies formed were observed and counted daily by the plate count method. The purification was carried out by putting the sample colony in 5 ml of sterile diluted  $\text{dH}_2\text{O}$  and 0.1 ml of dilution was poured in a petri dish containing three kinds of yeast extract mannitol agar (YEMA) media, namely YEMA, YEMA + Congo Red, and YEMA + Bromthymol Blue (BTB) media. The YEMA media was used as selection media for *Rhizobium*. YEMA + Congo Red was used to check the colony color that represents *Rhizobium*; the pink *Rhizobium* colony is a colony that did not absorb the congo red dye. YEMA and BTB were used to check the growth speed of the *Rhizobium*; the yellow-colored colony indicated a quick-growing colony, while the blue one did not. Isolated single colonies were grown in a slanted medium in a test tube as a pure culture.

***Rhizobium* activity test: Siderophores production.** Siderophores production was tested qualitatively using the chrome sulfate azurol (CAS) selective media and incubated at 27-28°C for 7 days. A positive result was indicated by the formation of clear zones around the bacterial colony (15).

**Phosphate solubilizing activity test.** Phosphate solubilizing activity was tested qualitatively using *Pikosvkaya* media as described in Gupta et al. (16) and incubated at 27-28°C for 2-7 days. A positive result was indicated by a halo zone production by a colony, indicating that the bacteria can solubilize phosphate.

**Protease production.** Protease production activity was tested qualitatively using a skim milk agar (SMA) as in Bhowmik et al. (17). The bacteria were

**Table 1.** *Rhizobium* isolates and the origins of samples

<i>Rhizobium</i> isolate	Origin of sample	<i>Rhizobium</i> population ( $\times 10^7$ CFU/g of soil)
1A and B	Peanut Dieng, Dieng (Wonosobo)	65
2A and B	<i>Solanum</i> sp., Dieng (Wonosobo)	103
3A and B	Soil crater of Sikidang 1, Dieng (Wonosobo)	40
4A and B	Soil crater of Sikidang 2, Dieng (Wonosobo)	35
5A and B	<i>Carica papaya</i> , Dieng Kulon (Banjarnegara)	77
6A and B	<i>Solanum</i> sp., Dieng kulon (Banjarnegara)	55
7A and B	<i>Solanum</i> sp. + mulch, no microsols (Banjarnegara)	81
8A and B	<i>Zea mays</i> , Wanaraja (Banjarnegara)	53
9A and B	<i>Solanum</i> sp + mulch + microsols 1 (Banjarnegara)	66
10A and B	<i>Solanum</i> sp + mulch + microsols 2 (Banjarnegara)	46
DW A and B	<i>Daucus Carota</i> , Wanaraja, Banjarnegara	37

inoculated and incubated at 28-30°C for 2-5 days. A positive result was a clear zone around the colony, indicating that these isolates were able to produce a protease enzyme.

**Indole acetic acid (IAA) production.** The IAA production activity was tested using TSB (tryptone soy broth) agar and incubated at 28-30°C for 2-5 days. Approximately 1 ml of *Salkowsky* solution was dropped on top of the growing colonies and then incubated in a dark condition for 1 hour. A positive result was noted by a color change to pink indicating that these isolates were able to produce IAA (18).

**The effectiveness of *Rhizobium* on peanut growth.** This study was conducted by a completely randomized design with three replications. The identified *Rhizobium* was labelled based on the origin area as 1B, 2B, 3B, 4B, 5B, 6B, 7B, 8A, 9A, 10A, DW 2, X (a mix of isolates 1B + 2B + 3B + 4B + 5B + 6B), and Y (a mix of isolates 7B + 8A + 9A + 10A + DW2) (Table 1 and 2) and then used as treatments. Each of these *Rhizobium* was inoculated in a peanut (*Arachis hypogaea* L.) growing medium by submerging the peanut in *Rhizobium* solution with  $10^6$ /mL CFU concentration. The controls were not inoculated and fertilized with N (K1) or fertilized with urea fertilizer equivalent to 100 kg/ha (K2). The plants were harvested after 40 days and the following parameters were observed: plant height (cm), leaf number, the dry weight of shoot (g), root (g), root nodules (g), and total plant (g). The measurement of chlorophyll index was done using SPAD-502 Plus (19). An average of 24% of soil humidity was maintained by water-

ing every day and using a nutrient solution without N (20).

**Data analysis.** The observed data were tested using a one-way analysis of variance (ANOVA) in 5% probability level to determine the significance of the treatment's effect on the observed parameters.

## RESULTS

**Characterization of *Rhizobium* isolate.** As shown in Table 1, the population of bacteria in the sampled soils and plants was 35 to  $10^3 \times 10^7$  CFU/g of soil. The samples taken from soil showed a smaller amount compared to the rooting areas. As shown in Table 2, four *Rhizobium* isolates grew slowly in YEMA media; 4 isolates had a moderate growth rate, and 3 isolates were fast-growing. Two slow-growing isolates were characterized by blue colonies in YEMA + Bromthymol Blue while 9 rapid growth isolates were a yellow-colored colony. The eleven isolates grown in YEMA + Congo Red media were all pink, indicating that the isolates were *Rhizobium*.

***Rhizobium* activity.** As shown in Table 2, 11 isolates of *Rhizobium* had high activity in promoting peanut growth, although the results varied considerably. Regarding plant nutrient availability, it was showed that 7 isolates could produce siderophores. Regarding the nutrient tests, nine isolates grown in protease media showed that they could produce protease enzymes. There were 7 isolates capable to form colonies in NFB (Nitrogen Free Medium) for N-fix-

**Table 2.** *Rhizobium* isolates characteristics

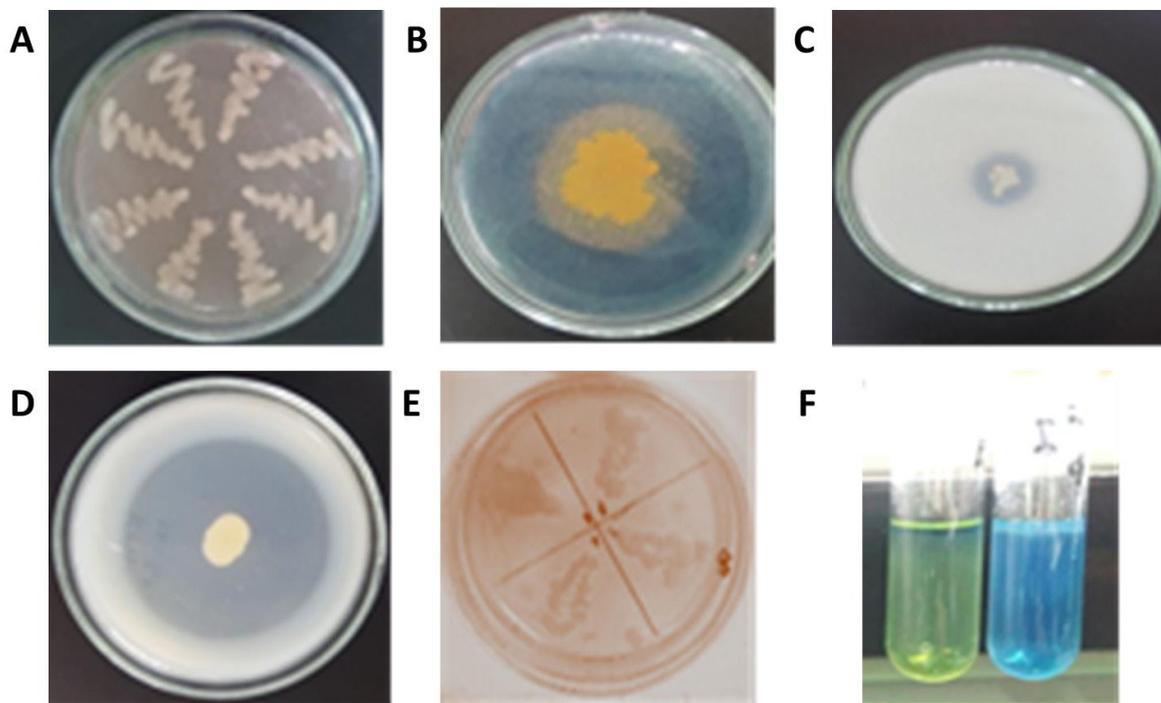
<i>Rhizobium</i> isolate	YEMA	YEMA + CR	YEMA + Growth	BTB Color	Activity test				
					Siderophores (cm)	PK (cm)	Protease (cm)	IAA	N-fixation
1B	+	P	F	Y	2.2	-	-	No	Yes
2B	++	P	F	Y	2.2	0.1	-	No	Yes
3B	+	P	S	B	3.4	-	1.7	No	Yes
4B	+	P	S	B	-	-	1.8	No	No
5B	+	P	F	Y	2.1	-	2.2	No	Yes
6B	+++	P	F	Y	3.4	0.2	1.8	No	No
7B	+++	P	F	Y	-	0.2	0.4	Yes	No
8A	+++	P	F	Y	-	-	2.3	Yes	Yes
9A	++	P	F	Y	1.1	0.1	2.8	Yes	No
10A	++	P	F	Y	1.0	0.1	2.5	Yes	Yes
DW2	++	P	F	Y	-	-	2.6	No	Yes

**Note.** YEMA: Yeast extract mannitol agar; CR: Congo red; BTB: Bromothymol blue; +: less fertile; ++: fertile; +++: high fertile; P: pink; F: fast; S: slow; Y: yellow; B: blue; PK: phosphate solubility; IAA: indole acetic acid production.

ation assay, indicated by the white ring or a pellicle formation below the media's surface and the change in the media color from a cream color to blue. The activities of the isolate were shown in Fig. 1.

**Peanut growth.** The result showed that the difference in *Rhizobium* isolates significantly affected pea-

nut growth and development ( $p < 0.05$ ). The highest plant yields were on plants inoculated with isolates 10A and Y. Isolate 1B gave the highest value in the number of leaves and the dry weight of canopy, root nodule, and total dry weight. The highest dry weights and chlorophyll index were found in plants inoculated with isolate Y. The Y treatments gave the best



**Fig. 1.** Colony activity test. (a) *Rhizobium* isolates in YEMA+CR. (b) qualitative test of Siderophore. (c) Phosphate solubilizing. (d) Protease. (e) IAA hormone. (f) N-Fixation of *Rhizobium* bacteria.

results in plant growth based on all observed parameters (Fig. 2).

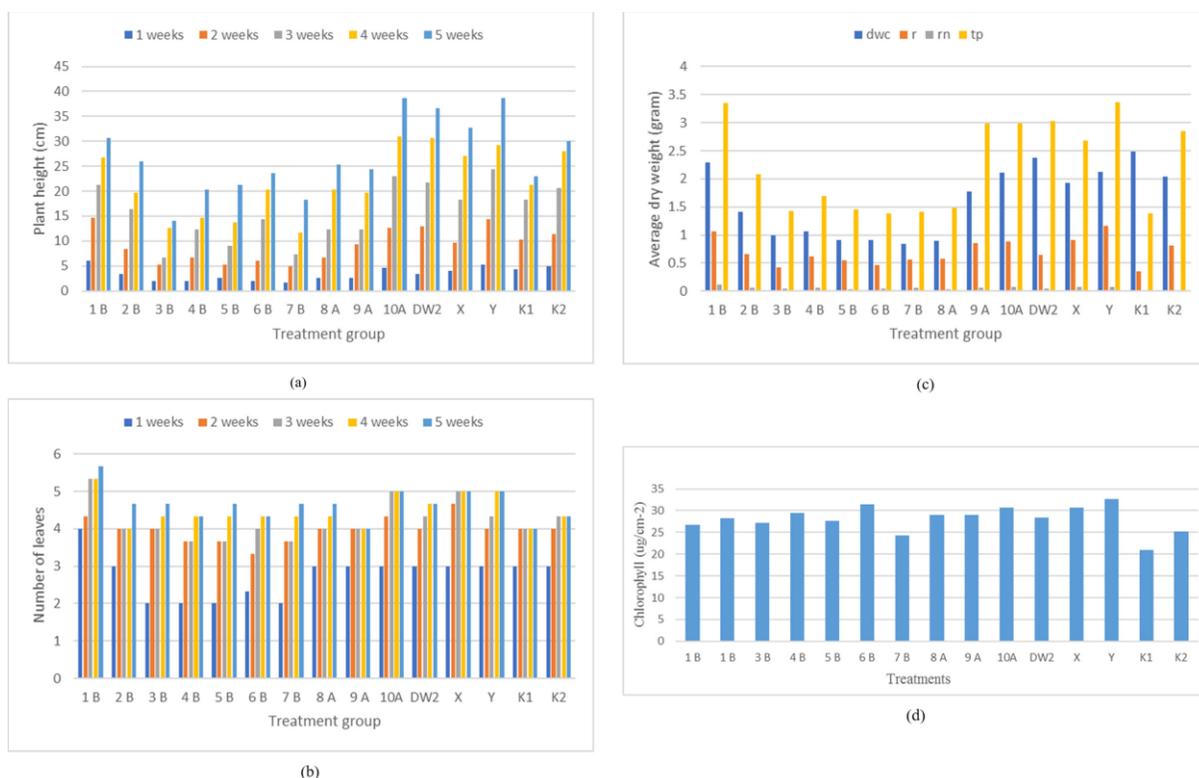
**DISCUSSION**

**Characterization of *Rhizobium* isolate.** The various *Rhizobium* population in Dieng indicated that there are different nutrient content in soil and on plant roots (11), which causes the different compositions and bacteria concentrations (21). The higher population density of *Rhizobium* bacteria in soil samples is likely due to the higher nutrients availability or the available nutrients are more suitable for *Rhizobium* growth. Dakora and Donald stated that root exudates consist of anions, sugar compounds, amino acids, organic acids, glycosides, nucleotide compounds and their bases, enzymes, vitamins, indol compounds, and others that can affect the population of bacteria in the soil (22). In other words, the metabolic activity and metabolite compounds released by plant roots into the soil determine the bacterial population. Also, the soil fertility factor, O<sub>2</sub> content, pH, nutrients as

well as physical and biological factors of soil affect the population of bacteria in the soil (23).

*Rhizobium* spp. bacteria can be identified from some selective media supplemented with specific dyes indicator. Our study observed that the *Rhizobium* sp. was dyed pink in YEMA + Congo Red media (Fig. 1). The result was following Somasegaran and Hoben's (24) statement, one of the characteristics of *Rhizobium* bacteria is that they are pink-colored on YEMA + Congo Red media because they do not absorb the red dye. BTB supplemented YEMA medium could be used to identify the bacterial ability to produce acidic or basic metabolite. The medium color will turn from red to blue if they produce basic substances or red to yellow for acidic substances (25).

***Rhizobium* activity.** In terms of helping plant nutrient availability, isolates that could produce siderophores may have an essential role in the host plant's survival. Siderophores are specific iron-defensive compounds, produced by microbes to sequester iron in the rhizosphere environment so that this element is not available to plants (26). The *Rhizobium* sp. iso-



**Fig. 2.** Peanut growth and development based on the given treatment. (a) Plant height and (b) the number of leaves observed from: (■): 1 week; (■): 2 weeks; (■): 3 weeks; (■): 4 weeks; (■): 5 weeks. (c) Dry weight of plant from: (■): dry weight of canopy (dwc); (■): root (r); (■): root nodule (rn); (■): total biomass dry weight (tp). (d) Chlorophyll content

lates grown in PK medium were characterized by the formation of halo zones around the colony, indicating that the bacteria could solubilize bound phosphate to provide phosphate for the plant (27).

Few isolates showed the capacity of producing protease enzymes and hydrolyzing proteins into simpler molecules, such as oligopeptides or amino acids, which are needed for plant growth (28). The isolates that were grown in TSB media for the IAA test showed nine positive, characterized by pink colony color. Such characteristics can help to influence many physiological processes, including cell enlargement and division, and produce more lateral roots and roots hairs, which aiding the plant to uptake the nutrients (29).

**Peanut growth.** Based on the peanut growth bioassay, the Y treatment that combined five isolates (7B, 8A, 9A, 10A, and DW2) induced the best growth promotion of peanut plants. The mixture of these strains could support the nutrient uptake since they were fertile, able to produce siderophores and hydrolyze protein as well as showing P-solubilizing effect. The inoculation had a significant effect on growth and yield, yet the level of effectiveness varies greatly depending on the symbiosis between the plant and the given inoculant (30).

*Rhizobium* inoculation plays an important role in providing nutrient N by forming root nodules, which bind atmosphere N into a readily-absorbed N. Besides, inoculation will have a significant effect on growth if there is a match between the inoculated *Rhizobium* and the host plant (31). Effective and efficient *Rhizobium* inoculation will able to compete with the indigenous *Rhizobium*, and able to synergize with its host plants (32).

The inoculated *Rhizobium* is expected to support plant growth, either directly or indirectly. The direct growth support by nitrogen fixation from the atmosphere, dissolving minerals like phosphate, production of siderophore, and the synthesis of growth hormones, such as IAA, gibberellin. On the other hand, the indirect ways were biocontrol of root pathogens, microbial destruction through the production of antibiotics, catalase, and siderophores (33).

## CONCLUSION

This study concluded that the best inoculants for

*Arachis hypogaea* L. growth were obtained from strain B1 of *Rhizobium* sp. The combination of several strains showed different beneficial characteristics. These isolates can be utilized to develop a formulation of biological fertilizer agents for peanuts.

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