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Original Article

Diversity, activity analysis and effectivity of rhizobacteria in plants rhizosphere on the growth of *Arachis hypogaea* L plants

Sri Purwaningsih*

Research Center for Biology, Indonesian Institute of Sciences, Indonesia.

Abstract

This research was conducted to observe the diversity, activity analysis and effectivity of rhizobacteria in plant rhizosphere on the growth of *Arachis hypogaea* L plants. The soil samples were collected from rhizosphere soil from different plants at Suro Muncang village and Bukit Peninjauan village. The rhizobacteria was isolated in YEMA media to test the qualitative activity including nitrogen, indole acetic acid (IAA) fixation, and siderophores. An isolate used in this research were 1 RB, 2 RB, 8 RB, 9 RB, 10 RB, 11 RB, 16 RB, 17 RB, 19 RB, 20 RB, 21 RB. Experimental plants were harvested after 45 days. The parameters used were the dry weight of shoots, roots, nodules root, total plants, number of nodules, and symbiotic capacity. The research design was conducted by Completely Randomized Design with three replications per treatment. The results showed that the population of rhizobacteria ranged between 13-51 x 10^5 CFU/g soil. Rhizobacterial isolates showed different level of plant growth promotion, which is the isolate 19 RB (isolate from *Leucaena leucacephala* plant) has a higher influence to the growth of *A. hypogaea* L.

Keywords: *Arachis hypogaea* L, biological fertilizer, rhizobacteria

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Introduction

Peanut (*Arachis hypogaea* L) is a crop having high economic value apart from soybean and mung beans. The demand for peanuts keeps increasing by a year. Domestic production is not cover the market need, thus import supply was needed. So, the production capacity need to be improved (Eman & Sumarno, 2014). Boosting the productivity of *Arachis hypogaea* L plants is conducted by improving the fertilization by using chemical fertilizer as well. However, the high expense of chemical fertilizer encouraged to find an alternative fertilizer that capable providing plant nutrients, such as biofertilizer. Biofertilizer, its low price, capable to maintain soil fertility, improve crop production and protect the environment (Gupta et al., 2015). Rhizobacteria is included as group of biological fertilizers.

Rhizobacteria are capable to live in the soil, within a root nodule and to grow in a symbiotic contact with leguminous or plants. These bacteria have a role in nitrogen fixation, growth hormones production, enzyme siderophores, proteases and other substances production. Rhizobacteria are capable to associate with plant roots such as living in the roots rhizosphere and living independently in the soil. These bacteria belong to a group of plant growth promoting bacteria called PGPR (Plant Growth Promoting Rhizobacteria (Aditya, 2009; Hemahenpagam, 2011), which can be used as a biofertilizer agent due to its ability providing nutrients for plants. Rhizobacteria are also able to supply growth hormone such as auxin, gibberellin and cytokinin that have significant effect in the rooting formation system and the siderophores enzymes production as biocontrol. The growth of rooting system would allow, plants, to find the sources of nutrients stimulating a growth and forming a resistance to various diseases (Tisha & Meenu, 2017; Yousefi & Barsegar, 2014).

Rhizobacteria are regularly found in the rhizosphere with various number per gram of soil. The factors affecting the population of rhizobacteria are the type of plant and fertilization, soil type, soil pH, carbon source, light intensity, a number of nitrogen-fixing bacteria in the roots, oxygen concentration and an extreme environmental factors (Subha & Rajesh, 2018). The isolation of rhizobacteria increased the availability of nutrients especially nitrogen (Sadam et al., 2018). The presence of rhizobacteria raised the production of corn and reduced the use of artificial fertilizers about 15-30% (Damir, 2011). Gomare (2013) stated that the administration of rhizobacteria increased the dry weight of shoot and roots. Rhizobacteria can be applied to agricultural soil as biofertilizer due to the activity of nitrogen fixation and growth hormones production that provide nutrients for plants (Himanshu et al., 2018).

To investigate the existence of Rhizobacteria, the data collection and rhizobacteria isolation had been conducted. This research was expected to obtain a pure culture having the ability in nitrogen fixation, producing growth hormone and siderophores. Thus, effective isolate which able to increase the growth plants could be developed as a biofertilizer improving soil fertility.

* Corresponding Author:
Sri Purwaningsih
Research Centre for Biology, Indonesian Institute of Sciences, Jl Raya Jakarta-Bogor Km 46 CSC, Cibinong, West Java, Indonesia
Phone: +62218765066 Fax: +62218765062
e-mail: sipur2005@yahoo.co.id

Methods

Materials

Composite soil samples were collected randomly at the depth of 0-15 cm at Suro Muncang and Bukit Peninjau villages. The media used for rhizobacterial isolation was YEMA (Yeast Extract Mannitol Agar) media consist-

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ing of 0,5 g K_2HPO_4, 0,2 g $MgSO_47H_2O$, 0,1 g NaCl, 3 g $CaCO_3$, 10 g Mannitol, 3 g Yeast extract, 20 g Agar, 1000 mL Aquadest, pH 6.8 (Vincent, 1982).

Isolation, Purification and Characterization of Rhizobacteria

The isolation was perfomed by serial dillution. 1 g of soil samples transferred onto 9 mL of physiological saline solution (0.85% NaCl) in a reaction tube then shaken by vortex mixer until 10^{-1}-10^{-5}. 0.1 mL solution was poured into a media and incubated at room temperature. The colonies formed were observed and counted daily, using plate count method (Somasegaran & Hoben, 1994). The isolates obtained was transferred to a slanted media, then purified to obtain a pure isolate. An isolate was transferred onto 5 mL aquadest and mixed with a vortex. 0.1 mL was poured in petri dish containing YEMA media, spread evenly with a spatula and incubated at room temperature.

Nitrogen Fixation Test

The pure isolates were inoculated on a NFB semi solid media (Nitrogen Free Brom Thymol Blue) (Dobereiner, 1991), consisting of 5 g Malic Acid, 4 g KOH, 0,5 g K_2HPO_4, 0,5 g $FeSO_47H_2O$, 0.01 g $MnSO_4H_2O$, 0.01 g $MgSO_47H_20$, 0.1 g NaCl, 0.02 g $CaCl_2$, 0.002 g $Na_2MoO_42H_2O$, 4 mL 1.64% Fe-EDTA, 4 g KOH, 1 mL Vit solution, 2 mL microelement, 2 mL BTB (0.5% alcoholic sol.), 22 g Agar and 1000 mL ddH2O. Bacterial isolates were grown in the reaction tube containing semi solid NFB media, and incubated at room temperature for 2-7 days. Nitrogen fixation was indicated by formation of a white ring on the surface of the media.

Indole Acetic Acid (IAA) Fixation Test

IAA production was analysed qualitatively using TSB (Trypton Soya Broth) media, consisting of 10 g Peptone, 2.5 g NaCl, 22 g Agar, 1000 mL Aquadest. Bacterial isolates were inoculated at the center of the petri dish consisting TSB media, and incubated at room temperature for 2-5 days. 1 mL Salkowsky solution (1 mL 0.5 M $FeCl_3$ + 50 mL 50% $HClO_4$) dropped onto growing bacterial colonies and incubated in a dark place approximately 1-3 h. IAA production was indicated by a formation pink color (Schroder, 2003).

Siderophores Production Analysis

The production of siderophores was conducted qualitatively by using selective media, Chrome Sulfate Azurol, framework of CAS Agar, consisting of blue dye (0.06 g Chome Azurol in 50 mL ddH_20), 0.0027 g $FeCl_36H_20$ (in 10 mM HCl), 0.073 g HDTMA (in 40 mL ddH_20), Gucose (20 g D Gucose in 100 mL ddH_20), Casamino Acid (5 g Casamino Acid in 45 mL ddH_20 + 1.35 g Hydroxyquinoline in 45 mL Chlorofom), MM9 media (5 g KH_2PO_4, 25 g NaCl, 50 g NH_4Cl in 500 mL ddH_20). CAS media (75 mL ddH_20 + 10 mL MM9 media + 3.024 g PIPES + 1.5 g Bactoagar) in autoclave) + 3 mL Casamino + 1 mL Glucose + 10 mL Blue Dye). Bacterial isolates were inoculated onto petri dish containing CAS agar media, then incubated at room temperature for 7 days. The production of siderophores was indicated by the formation of a clear zone around the bacterial colony (Schwyn & Neilands, 1978).

Rhizobacterial Effectivity Test

The potential isolates obtained were tested for their effectiveness on *Arachis hypogaea* L var. Zebra plant growth. The research was conducted in the greenhouse of Microbiology, Research Center for Biology, Indonesian Institute of Science by using sterile sand media, in 0.5 gallon plastic pots. The seeds were planted on a total 1.5 kg of sterile sand as growing media. Sterile sand mixed with paraffin and benzole (sterile) as high as 2 cm set as the seed cover. Rhizobacterial isolates used as inoculant were: 1 RB, 2 RB, 8 RB, 9 RB, 10 RB, 11 RB, 16 RB, 17 RB, 19 RB, 20 RB, 21 RB. Control plants treated without inoculation and N fertilizer (K_1), and without inoculation and supplemeted with N fertilizer equivalent to 100 kg/ha (K_2). The research design was conducted by Completely Randomized Design with 3 replications per treatment.

Plants were harvested after 45 days planted. The parameters observed were included plant height, number of leaves, shoot dry weight, roots, nodule, total nodule and total dry weight of plant. Watering (nutrient solution without bounded N) was conducted everyday to maintain moisture content (24%) (Saono et al., 1976). The ability of rhizobacterial isolate to form symbiosis contact was determined by Brockwell et al. (1965) as followed

Sc: (I-U) / (N-U)
Sc: Symbiotic capacity
I : average of shoot dry weight of inoculated plant
U : average of plant dry weight without inoculation and N (K_1)
N : average of plant dry weight without inoculation, added with N (K_2).

Sc scores were grouped into 4 categories: E (very effective) if Sc > 0.67, c (effective) if 0.33 < Sc < 0.67, e- (less effective) if Sc < 0.33 and I (not effective) if Sc < 0. In addition to Sc, the effectivity test could be performed by comparing the total dry weight of the tested plants to the dry weight of the control plant which was added with N (K_2) fertilizer, stated in percentage (Date in Vincent, 1982)

Results

The numbers of rhizobacterial population were varied in each plant root. Soil samples collecting from Suro Muncang village, Ujan Mas sub-district, Kapahiang district, Bengkulu province were 15 samples. The population of rhizobacterial varied from 14-56 x 10^5 CFU/g of soil. The highest number was found in the roots of Avocado (*Persian Americana* Mill) plants (Tab. 1). Soil samples collecting from Bukit Peninjauan village, Sukaraja subdistrict, Seluma district, Bengkulu province were 10 samples. The number of Rhizobacterial population varied from 13-42 x 10^5 CFU/g of soil. The highest number was obtained from root system of rice plant soil (*Oryza sativa*) (Tab. 2). The number of rhizobacterial isolate showing the ability of nitrogen fixation, IAA production, and siderophores production were 11 isolates (Tab. 3).

The potential isolates were assessed to *Arachis hypogaea* L observing plant growth (Fig. 1). The results indicated

that 11 rhizobacterial isolates were capable to form root nodules, which in turn improving plant growth (Figs. 2, 3 and 4). The analysis of symbiotic contact showed that all the rhizobacterial isolates were incredibly effective, therefore the isolates promoted plant growth (Tab. 4). An effectivity percentage of 19 RB isolate was the highest (Tab. 5).

Table 1. Rhizobacterial population in soil samples collecting from Suro Muncang village, Ujan Mas sub-district, Kapahiang district, Bengkulu province

Sample code	Root system	Population of rhizobacteria (CFUx10^5)/g of soil	Number of isolates
1 RB	Corn (Zea mays)	51	3
2 RB	Banana *(Musa paradisiaca*)	49	3
3 RB	Cassava (*Manihot utilissima*)	37	3
4 RB	Avocado (*Persia americana* Mill)	56	3
5 RB	Orange (*Citrus* sp.)	32	3
6 RB	Ambarella (*Spondias* sp.)	15	3
7 RB	Pepper (*Piper nigrum*)	43	3
8 RB	Melinjo (*Gnetum gnemon*)	19	3
9 RB	Gamal / Quick stick *(Glyciridia* sp.)	16	3
10 RB	Green Beans (*Vigna sinensis*)	26	3
11 RB	Coffee (*Coffea* sp.)	21	3
12 RB	Papaya (*Carica papaya*)	34	3
13 RB	Dadap/ Coral tree (*Erythrina* sp.)	27	3
14 RB	Rice (*Oryza sativa*)	14	3
15 RB	Protected forest from Saba Penanjung	14	3

Note: RB (Rhizobium from Bengkulu)

Table 2. Rhizobacterial population in soil samples collecting from Bukit Peninjauan village, Sukaraja sub-district, Seluma district, Bengkulu

Sample code	Root system	Population of Rhizobacteria (CFU X 10^5)/g of soil	Number of isolates
16 RB	Rice (*Oryza sativa*)	42	3
17 RB	Corn (*Zea mays*)	13	3
18 RB	Watermelon (*Citrulus lanatus*)	35	3
19 RB	White Lead Tree (*Leucaena leucocephala*)	18	3
20 RB	Banana (*Musa paradisiaca*)	22	3
21 RB	Without plant	26	3
22 RB	Oil palm (*Elais guinensis*)	33	3
23 RB	Sweet Potato (*Ipomea batatas*)	19	3
24 RB	Rubber Plant (*Hevea brasilensis*)	36	3
25 RB	Cassava (*Manihot utilissima*)	26	3

Table 3. The rhizobacterial isolate showing the ability of nitrogen fixation, IAA production, and siderophores production ion collecting from Suro Muncang village, Ujan Mas sub-district, Kapahiang district, Bengkulu

No	Isolate code	N-Fixation test	IAA production	Siderophores production
1	1 RB	+	+	+
2	2 RB	+	-	-
3	8 RB	+	+	+
4	9 RB	+	+	+
5	10 RB	+	+	-
6	11 RB	+	+	+
7	16 RB	+	-	+
8	17 RB	+	+	-
9	19 RB	+	-	+
10	20 RB	+	+	+
11	21 RB	+	+	+

Table 4. Nodule formation, number of nodules (nn), Symbiotic capacity (Sc) and effectivity percentage (PK) of rhizobacterial isolates inoculated to *Arachis hypogaea* L plants (45 days old)

Isolates code	Nodule formation	nn	Sc		PK (%)
			Real value	Relative value	
1 RB	+	88.33 bc	e.	0.50	86.97
2 RB	+	111.67 de	E	1.27	108.46
8 RB	+	116.00 de	e-	0.31	80.61
9 RB	+	118.33 de	E	1.02	101.46
10 RB	+	75.33 b	E	0.93	98.37
11 RB	+	105.67 de	E	1.05	101.95
16 RB	+	112.67 de	E	1.20	107.00
17 RB	+	76.66 b	E	0.70	91.04

19 RB	+	110.33 de	E	1.63	118.40
20 RB	+	96.66 cd	E	1.02	102.76
21 RB	+	113.33 de	E	0.90	99.02
K_1	-	0.00 a	0	0	0
K_2	-	0.00 a	0	0	0

Note: number followed by the same letter in every column showed no significant difference in Duncan Test at the 5% significance level

Table 5. The highest average and percentage of plant growth on plants inoculated with rhizobacterial isolates

Parameters	Isolate code (the highest) (g)	Growth Rise (%)
dwc (45 days old)	19 RB (6.94)	57.14
r (45 days old)	19 RB (0.36)	71.42
rn (45 days old)	21 RB (0.3644)	0
tp (45 days old)	19 RB (7.27)	18.40
nn (45 days old)	9 RB (118.33)	0

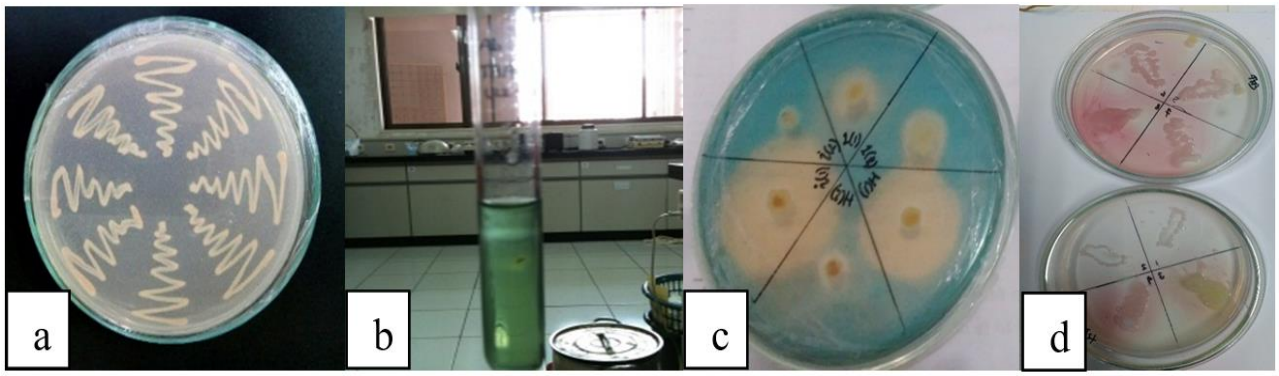

Figure 1. (a) The pure isolate in YEMA media, (b) Nitrogen Fixation, (c) Siderophore production, (d) IAA production.

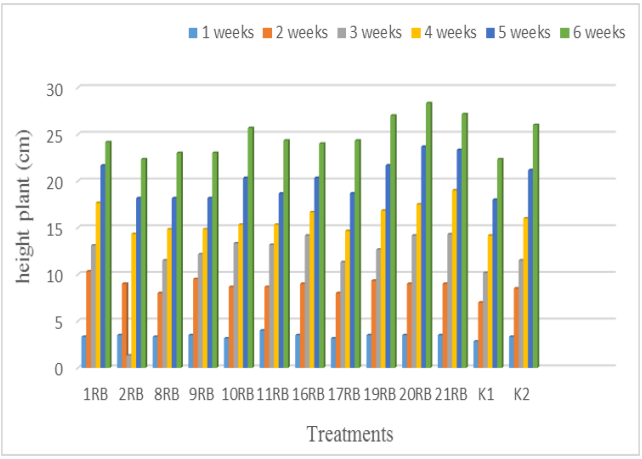

Figure 2. The average of plant height *Arachis hypogaea* L plants inoculated with rhizobacterial isolates (cm).

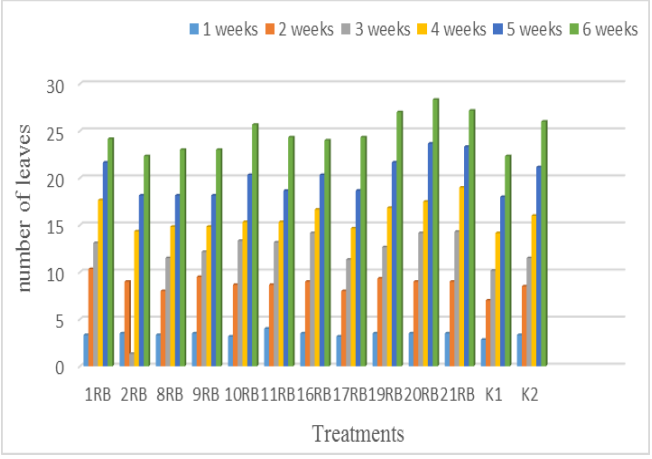

Figure 3. The average of leaves number *Arachis hypogaea* L plants inoculated with Rhizobacterial isolates.

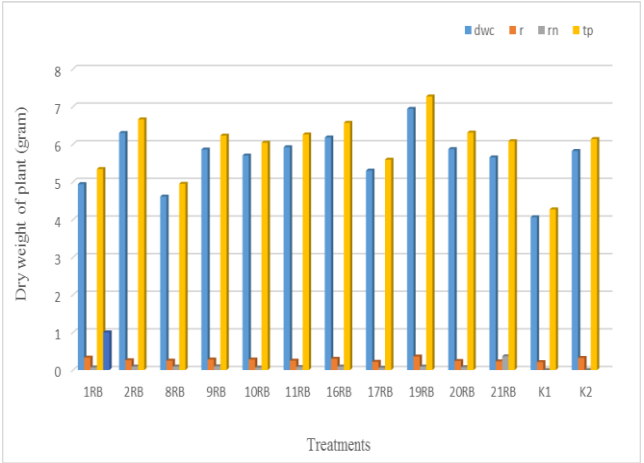

Figure 4. The average of dry weight of canopy (dwc), roots (r), root nodules (rn) and total plants (tp) of *Arachis hypogaea* L plants inoculated with Rhizobacterial isolates.

Discussion

The results of the study showing that the number of rhizobacterial population found in soil samples suround plants rooting system was higher than in soil samples without plants. It was due to the association of rhizobacteria with plants which bacteria consumed organic material or exudate released by plants through rooting as nutrients. Consequently, the numbers of rhizobacteria in the root area were plentiful. Rhizosphere is part of the soil with the highest metabolic activity defined as a small volume of soil directly influenced by root growth and

metabolism (Naz, 2012). Root exudates caused plants and microbes interacting and stimulating each other, while affecting the growth and activity of microbes in the rhizosphere, rhizoplan and its surroundings (Abera, 2015).

In addition, the rooting areas of plants were occupied by beneficial organisms utilizing organic substrates or plant exudates as energy and nutrients sources. Some microbes play an important role for soil health and indicators determining soil quality (Jamal, 2010; Hajnal et al., 2012). Kizilkaya (2009) stated that rhizosphere was the area where biological and chemical activity of the soil took place. Both activity were influenced by releasing substances from plant roots continuously which play as nutrients source for soil microbe. Soil microorganism increased the availability of plants nutrients in the process of organic materials decomposition. Rhizobacteria as plant growth promoting agents produces various growth hormones and organic acids needed in the development of root hairs to improve plant growth (Mark et al., 2016; Defez et al., 2016). It can be concluded that there was a mutually beneficial symbiotic relationship between the plant and the inoculated of rhizobacteria.

There were 75 Rhizobacterial isolates obtained (Fig. 1a). The isolates grown in semi-solid NFB media formed white ring beneath in the media surface (Fig. 1b). It suggested that the isolates had nitrogen-fixing ability. It implied that the isolates were capable to provide available nitrogen for plants to grow and to develop and to increase soil fertility (Pajeres & Bohhanan, 2016). Isolates grown in TSB media formed a pink color (Fig. 1d), it indicated that 31 isolates capable to produce IAA hormone. Bacterial isolate that produce high IAA hormones may control physiological processes include cell expansion and division. In addition, it also able to form more lateral roots and root hair. Thus, the roots were able to extract more nutrients from the soil which eventually increase the growth and yield of the plant (Chaiharn & Lumyong, 2011).

Isolates grown in siderophores media formed a clear zone. It indicated that 24 isolates were able to produce siderophores (Fig. 1c). Siderophores play an important role in the host plant resistance. Siderophores were specific iron-bearing compounds produced by microbes that supplied available Fe^{3+} for plants and micronutrients in the rhizosphere. Therefore, those nutrients were not available for the development of pathogenic microbes (Sivasakthivalen & Stella, 2012).

Among 75 isolates obtained, there 11 isolates showed a high activity (Tab. 3) inoculated against on *Arachis hypogaea* L plant growth. This phenomena showed that 11 rhizobacterial isolates able to increase growth. It indicated that these isolates may form effective symbiotic contact with *Arachis hypogaea* L plants. It was characterized by better vegetative growth compared to non-inoculated control plants and without N fertilizer addition (K_1). According to Pushp et al. (2011) an effective symbiotic ability is known when inoculated rhizobacterial isolates formed root nodules. It showed that the nitrogen fixation works well which in turn improves plant growth. Plant growth was observed based on required parameter including on the highest plant height and number of leaves which found in the plant inoculated with isolate 20 RB, on the highest dry weight of shoots, root and plant total value which found in the plant inoculated with isolate 19 RB, and on the measurement of dry weight of the highest rated nodule which found in the plant inoculated with isolate 21 RB.

Analysis on symbiotic capacity showed that the inoculated rhizobacterial isolates were very effective which resulted eventually in growth improvement. Parameswari et al. (2015) stated that excellent plant growth indicated the success of microbial inoculation. Well growth showed that microbial inoculated was able to form symbiotic contact with plants to increase growth.

Effectivity percentage was greatly affected by the characteristic of each isolate inoculated and suitability to the host plants. Effective and beneficial symbiosis contact will be established once the isolates fit and suitable for the host plants. Dasnadi et al. (2011) stated that there were differences in compatibility of symbiotic contact between isolates and host plants. Furthermore, it was also affected by physiological and environmental factors.

The observation of overall parameters showed that the isolate 19 RB had the highest plant growth in almost all observed parameters. It indicated that the isolate was effective and capable to form symbiosis contact with *Arachis hypogaea* L plants, and this isolate can be developed as a biological organic fertilizer agent, especially for *Arachis hypogaea* L plants.

The results of the study showed that the number of rhizobacterial population in both villages vary extensively. The number of bacteria ranged from 13 - 51 x 10^5 CFU/g of soil. The highest bacterial population were found in the root system of corn crops (*Zea mays*). There were a total of 75 isolates obtained, 75 isolates had nitrogen fixation ability, 31 isolates were able to produce IAA hormone and 24 isolates were able to produce siderophores. Isolate number 19 RB presented the highest plants growth on *Arachis hypogaea* L. Such isolates can be developed as biological fertilizer agents, especially *Arachis hypogaea* L plants.

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