Isolation and Characterisation of *Rhizobium* and *Rhodospirillum* species From Soil Samples of Kan Gyi Daunt Township And their Effects on The Growth of *Vigna Mungo* L.(Black Gram)

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Soil samples were collected from two different areas of KanGyiDaunt Township, Ayeyarwady Region. These samples were cultured on *Rhizobium* medium and Centenummedium. A total of sixteen bacterial colonies were obtained from these soil samples. Among them, seven bacterial isolates were obtained from *Rhizobium* medium and these strains were designated as Rhi-1 to Rhi-7. Other nine bacterial strains were obtained from Centenum medium and these strains were given as Rho-1 to Rho-9. The isolated bacteria were verified and characterised by morphological as well as with some biochemical tests. In the colony morphology, the sizes ofisolated bacteria were medium (4-5 mm) and large (6-24 mm) in size. The margin of colonies was entire, lobate, undulate and rhizoid. The morphological study was carried out by identifying their colony patterns on their culture medium and the shape of cells was determined by the Gram staining.In the Gram staining reaction, Rhi 1 to 7 were Gram negative, short rod and Rho 1to 9 were Gram negative and spiral shape.Biochemical tests were employed for characterization of isolated bacteria. Altogether 15 items of the following biochemical tests; catalase test, soluble starch, rice powder, tapioca, wheat, glutinous powder as starch hydrolysis activity tests, H_2S and motility test, citrate test, MR test, sugar fermentation,1%, 3% and 5% of salt tolerance and potato slice test were undertaken to identify isolated bacteria. According to the results, Rhi 1 to Rhi 7 were characterised as *Rhizobium* sp. Other bacterial strains were identified as *Rhodospirillum*sp. In the biofertilizer effect, both these bacteria were evaluated base on their ability to promote growth of *Vignamungo* (Black Gram). In germination test, seeds of Black gram treated with the bacterial broth increased significantly seed germination. Fresh and dry weights of the whole plant compared to the control and there were outstanding in the root depth and seedling height.

Key Words: *Rhizobium*, *Rhodospirillum*, Biochemical Tests, Germination Effects

Introduction

A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment where diversity is low (Graci*et al*., 2011). Since, bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to a greater extent, especially considering their competitiveness in root colonization (Barriuso*et al*., 2008).

Rhizobia are the gram-negative bacteria which have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen (Teaumroong and Boonkerd, 1998). These inhibit the root nodules of most legumes which can provide enough nitrogen for their physiological needs (Cleyet-Marel*et al*., 1990).

*Rhodospirillum*bacteria is Gram-negative, motile, spiral shaped bacteria. They can grow under many different types of conditions including aerobic or anaerobic environments. *Rhodospirillum*was found to be most efficient and produce the maximum levels of internal photosynthetic membranes when it was grown with both

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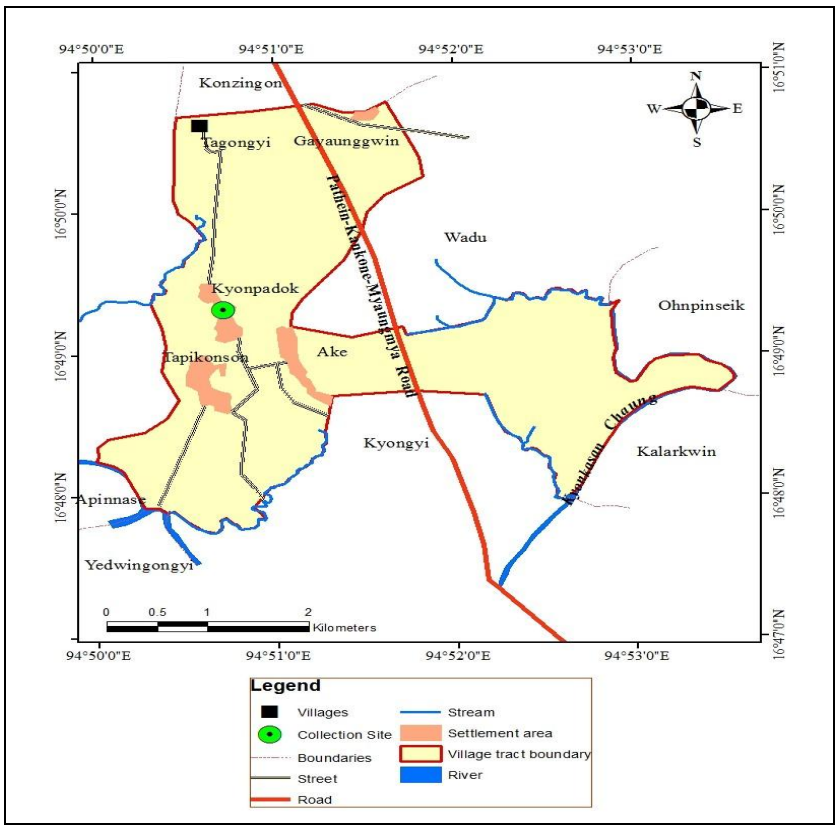
succinate and fructose as carbon sources under microaerophilic conditions (Grammel*et al*., 2003).

The aim and objectives of the present research work are to isolate the *Rhizobium*sp and *Rhodospirillum*sp from soil of KanGyi Daunt Township, Ayeyarwady Region, to study the colony morphology of isolated soil bacteria,to observe the shape of bacterial cells, to characterise the isolated bacteria as *Rhizobium*and *Rhodospirillum*by using the biochemical tests and to recognize the effects of *Rhizobium* and *Rhodospirillum* on the growth of *Vignamungo.*

Materials and Methods

Collection of Soil Sample

Soil samples were collected from the rhizosphere soil of Blackgram field and muddy soil (94˚50̊ E, 16˚49 N) of KanGyi Daunt Township, Ayeyarwaddy Region. These samples were cultured on Rhizobium medium and Centenum medium.



Source: Department of Geography, Pathein University

Fig.1. Location Map of Collected Soil Samples

Isolation of *Rhizobium* and *Rhodospirillum*Bacteria from Soil Sample

Serial dilutions of fermented, plating and Streaking techniques described by Salle (1948), Collins (1964) and Pelezer and Chan (1972) were used for the isolation of *Rhizobium* and *Rhodospirillum*bacteria isolates from soil sample. An appropriate amount (1gm) of soil was introduced into a conical flask containing 99 mL of distilled water to make a soil-water dilution ratio of 1:100. The flask was then shaken for about 30 minutes in order to make the soil particles free from each other. This solution was then serially diluted into 10^3 to 10^7 dilutions in separate test tubes and 1 mL each of the above dilutions was separately transferred into sterile petridishes under aseptic

condition. A sterile pipette was used for each transfer. An appropriate amount (10mL) of the medium was separately into test tubes and pugged with non-absorbent cotton wool. They are sterilized by autoclaving at 15 pounds pressure per square inch for 15 minutes. The sterilized medium in each conical flask was cooled down to about 45˚C and separately poured into the petridish containing the respective soil sample dilutions. The inoculated plates were shaken clock-wise and anticlock-wise direction for about 5 minutes so as to make uniform distribution of the bacterial inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at 30˚C for 24 hours. Various types of colonies developed on the inoculated plates were separately streaked over another set of petridishes containing the same sterile *Rhizobium* medium and Centenum medium cultured repeatedly so as to obtain a pure culture of *Rhizobium* and *Rhodospirillum*strains. The isolated strains were maintained in *Rhizobium* and Centenum media for further experimentations.

Preparation of Culture Medium

***Rhizobium* medium (Atlas, 1993)**		**CentenumMedium(Atlas, 1993)**	
Yeast extract	10.0 g	Yeast extract	10.0 g
K_2HPO_4	0.5 g	Sodium pyruvate	2.2 g
$MgSO_4$0.2 g	K_2HPO_4	1.0 g	
NaCl	0.2 g	$MgSO_4$	0.5 g
$FeCl_3$	0.002 g	Vitamin B12	0.02mg
Agar	15.0 g	Agar	20.0 g
Distilled water	1000 mL	Distilled water	1000 mL

Staining Reactions of *Rhizobium* and *Rhodospirillum*Bacteria from Soil Samples

A drop of sterile distilled water was placed on clean glass slide and a small loop of isolated bacteria was smeared on the slide and allows it to dry. The smear was fixed by passing the dry side 3 or 4 times rapidly over a flame. The slide was covered with crystal violet stain and allow it to act for 1 minute. Then, the slide rinsed with distilled water for a few seconds. The slide was covered with fresh iodine solution and allow it to act for about 1 minute. The alcohol was added drop by drop and stop adding alcohol when no more colour flows out from the smear. For a thin smear 5-10 seconds may be for complete decolourization of Gram negative bacteria. As a counter stain, the smear was covered with safranin for about 1 minute and washed with distilled water. Then, the slide was placed at air dry. The stained slide was examined under the oil immersion objective of the microscope (Atlas, 1993).

Germination Test for the Vegetable Seeds and Evaluation of Proper Dilution Ratio

In the sterilized Petri dishes, about 200 seeds of Black gram seeds free from any damage were surface sterilized by rinsing in 95% alcohol for 3 minutes. They were then washed with sterile distilled water for 6 to 10 times. Surface sterilized seed was put into the petridishes which contained and autoclaved a cotton fabric and ratio of broth 5:5 (broth : DW) were poured in each petridish of isolated bacterial ratio was added. The plates were then, incubated at room temperature by covering a sheet of black paper over the plates. The germination percentage was visually checked daily up to seven days. The fresh weight and dry weight of germination in the tested petridishes were also measured in seven days.

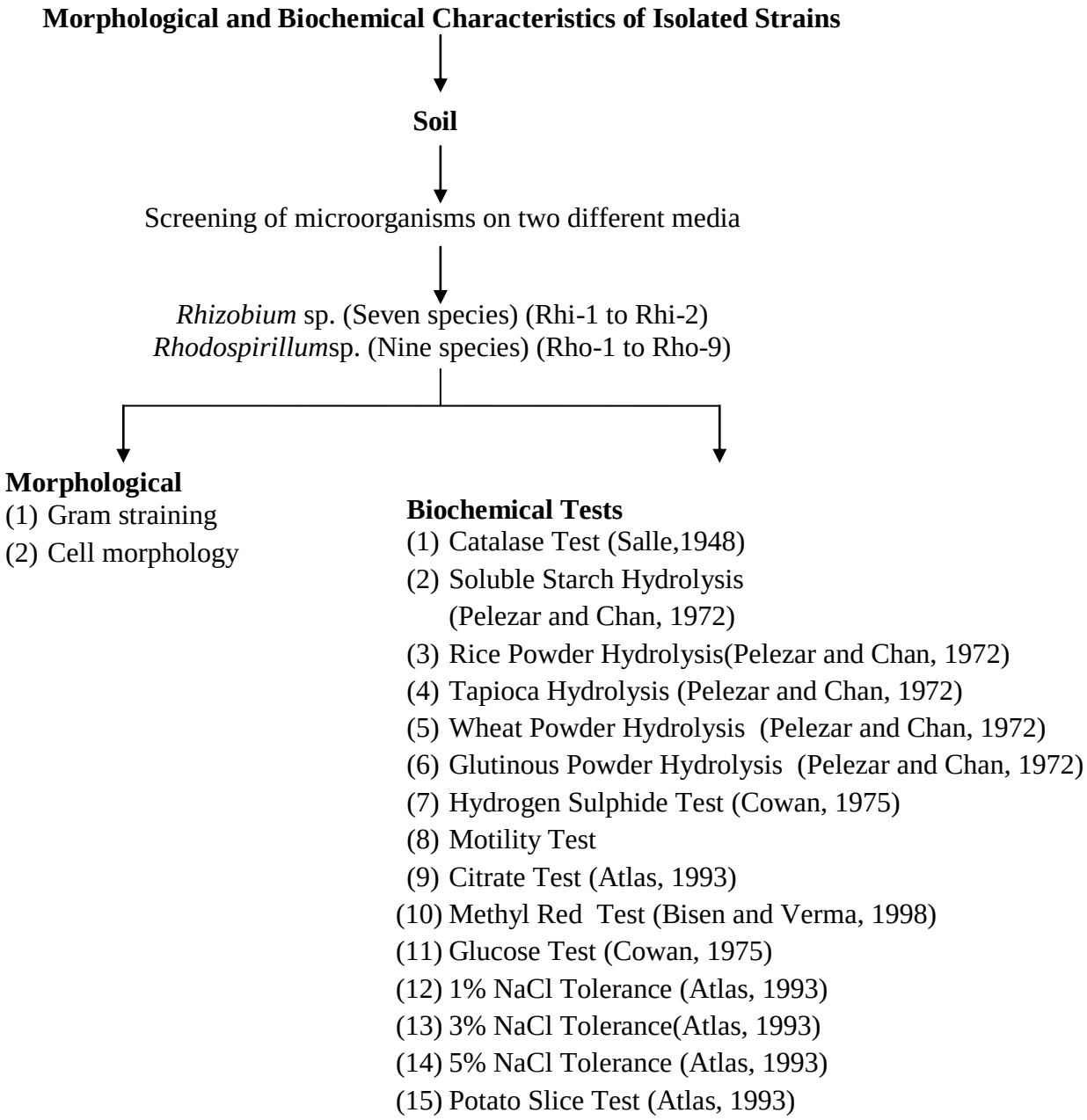


Fig.2. Morphological and Biochemical Tests for Isolated Bacteria *Rhizobium* and *Rhodospirillum*sp.

Results

Isolation of Bacteria from Soil

In the present study, *Rhizobium* and *Rhodospirillum*bacteria were isolated by serial dilution method from soil. A total of sixteen bacterial strains were obtained from two specific medium. Seven strains were from *Rhizobium* medium and nine isolates were from Centenummedium. (Table-1)

Identification of Isolated Bacteria *Rhizobium* and *Rhodospirillum*sp

In the microscopical and biochemical characters, Rhi 1 to Rhi 7 were short rod and Gram-negative, catalase positive and can hydrolyse the soluble starch, rice powder, tapioca, glutinous and wheat powder except for soluble starch, motility occurred and H_2S was produced, citrate and Methyl Red test were positive, acid and gas production from glucose sugar but Rhi-6 was not produced gas, grown in 1%, 3% and 5% Sodium chloride salt tolerance and potato slice. Therefore, these strains were in the group of *Rhizobium* formation. (Table 2, 4 and6)

In the microscopical and biochemical characters, Rho 1 to Rho 9 were spiral and Gram-negative, catalase positive and can hydrolyse rice powder, tapioca, glutinous, wheat powder and soluble starch, motility was and H_2S positive except Rho-8, both acid and gas were produced in the sugar fermentation, grow in 1%, 3% and 5% Sodium chloride salt tolerance and potato slice. These results were mentioned as the group of Genus *Rhodospirillum.*(Table 3,5 and 7)

Moreover, *Rhizobium* and *Rhodospirillum* were used as fertilizer and this study was observed by the addition of isolated bacterial strains (Rhi 1to 7 and Rho 1 to 9) to the experimental plants increased their growth over the control. In this study, the seeds of *Vignamungo* L.(Black gram) were cultivated by all treatments (5:5) of the bacterial strains in the petridish. After one week, the data was collected. The germination percentage of seeds of *Vignamungo* L. gave the best results in all isolated strains. In the seedling height, Rho-4 showed the tallest height (30cm) followed by Rhi-2 (27cm). Fresh weight and dry weight of the whole plant showed the effected influence of *Rhizobium* and *Rhodospirillum* on plant growth. (Table 8 and Figure 3)

Table.1. Isolated Bacteria on Selected Medium

No	Media	Isolated Bacteria
1	*Rhizobium* medium	Rhi 1 to 7
2	Centenum medium	Rho 1 to 9

Table 2.Colony Morphology of IsolatedBacteria on Rhizobium Medium

Medium	Shape	Elevation	Margin	Size	Pigment	Optical
Rhi-1	Circular	Raised	Entire	Large	Cream	Opaque
Rhi-2	Circular	Raised	Entire	Medium	White	Opaque
Rhi-3	Circular	Flat	Entire	Medium	Cream	Opaque
Rhi-4	Circular	Raised	Entire	Small	Pigment (Yellow)	Opaque
Rhi-5	Circular	Flat	Entire	Large	Pigment (Green)	Opaque
Rhi-6	Circular	Raised	Entire	Small	white	Opaque
Rhi-7	Circular	Flat	Entire	Large	Non-pigment	Opaque

Small = < 2mm Medium = 2mm-5mm Large = > 5mm

Table 3. Colony Morphology of IsolatedBacteria on Centenum Medium

Medium	Shape	Elevation	Margin	Size	Pigment	Optical
Rho-1	Circular	Raised	Entire	Large	Non-pigment	Opaque
Rho-2	Filamentous	Flat	Filamentous	Large	Non-pigment	Opaque
Rho-3	Circular	Raised	Entire	Large	Non-pigment	Opaque
Rho-4	Irregular	Flat	Undulate	Large	Non-pigment	Opaque
Rho-5	Irregular	Flat	Lobate	Large	Non-pigment	Opaque
Rho-6	Irregular	Raised	Undulate	medium	Non-pigment (cream)	Opaque
Rho-7	Irregular	Raised	Undulate	Large	Non-pigment (cream)	Opaque
Rho-8	Circular	Raised	Entire	Medium	Pigment (Green)	Opaque
Rho-9	Circular	Raised	Entire	Small	Non-pigment (white)	Opaque

Small = < 2mm Medium = 2mm-5mm Large = > 5mm

Table. 4. Cell Morphologyof *Rhizobium* Bacteria

Strain No.	Gram Staining	Cell Morphology
Rhi-1	-	Short rod
Rhi-2	-	Short rod
Rhi-3	-	Short rod
Rhi-4	-	Short rod
Rhi-5	-	Short rod
Rhi-6	-	Short rod
Rhi-7	-	Short rod

(-) = Gram negative

Table 5.Cell Morphologyof *Rhodospirillum*Bacteria

Strain No.	Gram Staining	Cell Morphology
Rho-1	-	Spiral
Rho-2	-	Spiral
Rho-3	-	Spiral
Rho-4	-	Spiral
Rho-5	-	Spiral
Rho-6	-	Spiral
Rho-7	-	Spiral
Rho-8	-	Spiral
Rho-9	-	Spiral

(-) = Gram negative

Table 6. Biochemical Characteristics of Isolated *Rhizobium* sp.

Biochemical Tests		Rhi-1	Rhi-2	Rhi-3	Rhi-4	Rhi-5	Rhi-6	Rhi-7
Catalase test		+++	+	++	+++	++	+	+
Starch Hydrolysis		+++	++	+++	+++	+++	+	-
Rice powder Hydrolysis		+++	++	++	+++	++	++	+
Tapioca Hydrolysis		+++	+++	+++	++	+++	+++	++
Glutinous powder Hydrolysis		+++	++	+++	+++	++	+++	++
Wheat Hydrolysis		+++	+	++	+++	+++	++	+++
Hydrogen Sulphide		+	++	+++	++	++	+	++
Motility test		+	++	+	++	++	+	+
Citrate test		+++	+++	+++	+++	+++	+++	+++
Methyl Red test		+++	++	++	+	++	++	++
Glucose Fermentation	Gas	+	+++	+++	++	+++	-	+++
	Acid	+++	+++	+++	+++	+++	+++	+++
NaCl tolerance 1%		++	++	++	++	+	+	+
NaCl tolerance 3%		+	++	++	+	++	++	++
NaCl tolerance 5%		+++	++	+	++	+++	+	+
Growth on Potato Slice		+	+	+	+	+	+	+

(+) = positive reaction (++)medium reaction (+++)high reaction (-)nonreaction

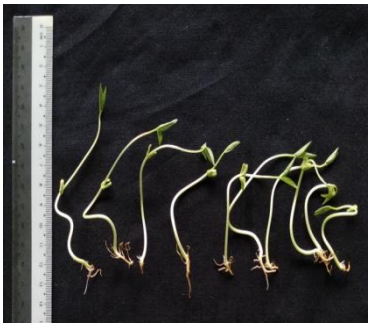
Table 7. Biochemical Characteristics of Isolated *Rhodospirillum*sp.

Biochemical Tests		Rho-1	Rho-2	Rho-3	Rho-4	Rho-5	Rho-6	Rho-7	Rho-8	Rho-9
Catalase test		+++	++	++	+	+++	+	+	++	+++
Starch Hydrolysis		+++	+++	+++	+	+++	+++	+++	+++	+++
Rice Hydrolysis		++	+++	++	+++	+++	+	++	+++	++
Tapioca Hydrolysis		+++	+++	++	++	+++	++	++	++++	+++
Glutinous Hydrolysis		++	+++	+++	++	+++	++	+++	+++	+
Wheat Hydrolysis		+++	+++	++	++	++	++	+++	+++	+++
Hydrogen Sulphide		++	++	+	++	+	++	++	+	+
Motility test		++	++	+++	++	++	++	++	++	++
Citrate test		+++	+++	+++	+++	+++	+++	+++	+++	+++
Methyl Red test		++	++	+	++	+	+	+	-	++
Glucose Fermentation	Gas	++	++	++	+	++	++	++	+++	++
	Acid	+++	+++	+++	+++	+++	+++	+++	+++	+++
NaCl tolerance 1%		++	++	+++	+++	+	++	++	++	++
NaCl tolerance 3%		+	++	++	+	++	+++	+++	+	+
NaCl tolerance 5%		+	+	+	+	++	++	+	+	+
Growth on Potato		+	+	+	+	+	+	+	+	+

(+) = positive reaction (++)medium reaction (+++)high reaction (-)non reaction

Table 8.Effects of fertilizer of Isolated Strains on the Germination of*Vignamungo*L. (Black Gram) in Petridish

Isolated Bacteria	Germination %	Seedling Height (cm)	Fresh Weight (g)	Dry weight per after 7 days (g)	Water content (g)
Control	80%	9.5	1.8	0.7	1.1
Rhi-1	100%	18	3.1	0.95	2.15
Rhi-2	100%	27	3.6	0.96	2.64
Rhi-3	100%	26	3.5	0.93	2.57
Rhi-4	100%	17	2.6	0.85	1.75
Rhi-5	100%	18.6	2.7	0.85	1.85
Rhi-6	100%	22	2.7	0.86	1.84
Rhi-7	100%	22	3.3	0.81	2.49
Rho-1	100%	23	3.4	0.93	2.47
Rho-2	100%	27	2.7	0.95	1.75
Rho-3	100%	24.5	3.3	0.85	2.45
Rho-4	100%	30	2.8	0.86	1.94
Rho-5	100%	21	3.2	0.96	2.24
Rho-6	100%	20.5	3.1	0.9	2.2
Rho-7	100%	21	2.7	0.8	1.9
Rho-8	100%	23	2.9	0.85	2.05
Rho-9	100%	23	4.1	0.9	3.2



Control

Rhi-2

Rhi-3

Seedling

Rho-2

Rho-4

Fig. 3. The Best Seedling Growth Effects of *Rhizobium*and*Rhodospirillum* Bacteria on the Germination of *Vignamungo*(Black gram)

Discussion and Conclusion

Bacteria are able to exert positive effects on plants through various mechanisms. Some bacteria solubilize insoluble minerals through the production of acids, increasing the availability of phosphorous and other nutrients to plants in deficient soils. Several bacteria improve plant growth through suppression of pathogens by competing for nutrients, by antibiosis, or by synthesizing siderophores which can solubilize and chelate iron from the soil and inhibit the growth of phytopathogenic microorganisms (Caballero, et al., 2007).

In the present study, two kinds of bacteria were isolated from soil. Seven bacteria were obtained from *Rhizobium* medium and other nine strains from Centenum medium. The isolated bacteria were identified by the morphological, cultural and biochemical characteristics. These characteristics were performed according to the Bergey's Manual of Determinative Bacteriology by Breed et al., (1957).

The isolated bacteria (Rhi 1 to 7) obtained from *Rhizobium* medium were circular, entire, flat and raise. Their colony color was cream, white and yellow. In the Gram reaction (Rhi 1 to 7) were Gram-negative and short rod and hydrolysed on various starch sources such as soluble starch, rice powder, tapioca, glutinous powder and wheat powder. However, Rhi-7 has not hydrolysed the soluble starch. These bacterial strains were positive in catalase, citrate, methyl red, H_2S and motility tests. Moreover, these bacteria also grow in 1%, 3% and 5% Sodium chloride salt tolerance and on potato slice respectively. In the sugar fermentation, Rhi 1 to 7 were fermented with glucose and acid and gas produced except Rhi-6. These characters were similar to the investigation on Genus *Rhizobium* of Buchanan, 1974. Therefore, Rhi 1 to 7 were classified as the Genus *Rhizobium.*

Other strains (Rho 1 to 9) were circular, irregular and filamentous in the shape. Their colony colors were cream and white. In the Gram reactions, these strains were Gram-negative and spiral shaped. In the biochemical tests, Rho 1 to 9 can hydrolyse on various starch sources. These bacterial strains were also positive in catalase, motility present, H_2S was produced, citrate test and MR test was positive except Rho-8, acid and gas was produced in glucose fermentation, can grow in 1%, 3% and 5% NaCl tolerance test and grown on potato slice. These results were identical to those of Pfennig (1971) and they were characterized as Genus *Rhodospirillum.*

Above all the results were matched with the descriptions of Bergry's Manual of Determinative Bacteriology by Breed, et al., 1957. Moreover, isolated bacterial strains *Rhizobium* and *Rhodospirillum* were used as biofertilizer and this study indicated the addition of these strains to the experimental crop plant increased their growth over the control. In the germination test of *Vignamungo* L., Rho-4 and Rhi-2 showed the highest seedling height. Narayanan et al., (2017) revealed that plant height is an important criterion for any crop in providing more places for flower production leading to better pod and fruit production. The initial vigour of the treated seeds might have helped to induce the seedling growth and increasing plant height with increased number of branches.

The use of PGPR differs on attractive way to replace chemical fertilizer, pesticides and supplements; most of the isolates result in a significant increase in plant height, root length and dry matter production of shoot and root of plants. PGPR help in the disease control in plants. Some PGPR, especially if they are inoculated on the seed before planting, are able to establish themselves on the crop roots (Kloepper*et al*., 2004).

In the fresh weight and dry weight, Rhi-2 and Rho-9 gave the moderate results. Takebe et al., (1995) reported that increments in leaf dry weight may be due to a combination of nitrogen with plant matter produced during photosynthesis.

It was concluded that the present study was the isolation of the bacteria *Rhizobium* and *Rhodospirillum*, characterization of the isolated bacterial strains and observation on the effected of the isolated bacterial strains on the growth of *Vignamungo* L. (Black gram).

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