Comparative Analysis between isolated PGPR species from *Rhizophora apiculate* and *Rhizophora mucronate*

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Abstract. The isolated PGPR strains from two different mangrove species, *Rhizophora apiculata* and *Rhizophora mucronata*, showed a potential biofertilizers for soyabean plant. These isolates exhibited various PGPR traits, including the ammonia and Indole acetic acid (IAA) production, with members of *Pseudomonas sp* and *Bacillus sp* among the diverse PGPR identified species. Particularly, *R. apiculata* showed superior PGPR characteristics which significantly enhanced the growth of soyabean plant as compared to *R. mucronata*. After 4 weeks of planting, the height and weight for *R.apiculate* were recorded 103.5cm and 130.21gm, respectively.

Keywords: PGPR Bacteria, *Rhizophora apiculate*, *Rhizophora mucronate*, Soybean Plant

1.0 Introduction

Sustainable agriculture consists of an integrated system which encompasses of various management procedures works with all natural resources to conserve agroecosystem, minimize hazardous environmental impact, sustain crop production. The chemical fertilizers produce greenhouse gas, soil and water contaminates, as well imbalance of nutrients in soil which alternatively affects the free-living organism in water. There are number of chemical fertilizers which are currently being used from past years destroying the soil biodiversity and ecosystem. People are generally concerned about chemicals used in agriculture. Thus, there has been a growing demand of biofertilizers products [1-3].

Biofertilizers are organisms which encompasses of plant growth promoting traits by the help of various nutrients. There are numerous microbes serve bio fertilizing activity such as bacteria, fungi, and cyanobacteria (blue-green algae) [4]. These diverse range of microbes consist of various genera such as *Bacillus, Pseudomonas, Klebsiella, Azotobacter* [5].

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PGPR plays an important role in sustainable agriculture system via managing plant growth and development by increasing high yield crop production which are cost effective, will help in decrease emission of various greenhouse gases, reduction in nitrogen leaching, and inhibit soil and water pollution [6]. Soybean scientifically known as *Glycine max L*. It is a significant source of food, protein, and oil [7]. It plays major role in vegetable oil production as well as animal protein feed [8]. In addition, soybeans are also used in biofuel production, aquaculture and contribute in human diet because of high protein content [9].

This research focused on isolated Plant Growth Promoting Rhizobacteria (PGPR) from two different pot plants *R. apiculata* and *R. mucronate*. The isolated strains were analyzed on their morphological, Gram staining, PGPR traits, and their effects on Soyabean plant growth.

2.0 Methodology

2.1 Sample Collection

The sample were collected from Sime Darby research center in Cary Island. The pot plants were collected from Sime Darby and preserved at 20°C.

2.2 Isolation of PGPR Strain

The isolation of PGPR strain was done by using a serial dilution method, by using 0.9% of NaCl in a 100 ml of distilled water and sterile in autoclave at 121°C for 15 minutes. The tryptic soy agar (TSA) media was used to grow the culture, the culture was incubated for 2 days at 30°C. The sub culturing was done weekly until the pure colonies were obtained.

2.2.1 Morphological Analysis of PGPR Isolated Strain. Once the isolated strain was obtained in pure colonies with no contamination, the morphological analysis was conducted on isolated strains. The macroscopic analysis contains the colony morphology of color/pigmentation, colony size, margin, and opacity.

2.2.2 Gram Staining. The Gram staining contains various stains such as crystal violet, Iodide, decolorize stain and safranin. The Gram-negative bacteria will show pink or red color. Whereas Gram-positive will show blue or purple color.

2.3 PGPR Characterization of Isolates Strain

2.3.1 Qualitative and Quantitative Analysis of Ammonia Production Test. Fresh culture was inoculated in 10 ml peptone water and incubated in a rotary shaker in room temperature for 48 to 72 hours at 120 rpm, the grown colonies were centrifuged for 10 minutes at 10,000 rpm. The 0.5 ml Nessler's reagent was added in 0.5 ml of culture media to study the color changing, color change results were further tested on UV-Vis spectrophotometer (CARY 5000) at 450 nm absorbance to observe the quantity of ammonia in each sample. The standard curve was made by using the different concentration of ammonia sulphate.

2.3.2 Qualitative and Quantitative Analysis of IAA Production Test. The IAA analysis was done by growing the colonies in the Tryptic S oy Broth (TSB) in an aseptic condition for 72 hours at room temperature for 120 rpm, grown colonies were centrifuged for 10 minutes at 10,000 rpm. The supernatant was collected were added in the double volume of Salkowski reagent in ratio of 2:1. The mixture was incubated in dark condition for 30 minutes. The color changes were further analyzed by using UV-Vis Spectrophotometer (Cary 5000) at wavelength of 530 nm. The standard curve was made by using the different concentration of IAA.

2.4 Soybean Plant Growth Analysis Under Isolated PGPR Strain

2.4.1 Bacterial Inoculum Preparation. Bacterial inoculum was prepared via using tryptic soy broth for 72 hours at room temperature for 120 rpm in 150 ml of TSB. The preparation ~108 CFU/ml of single PGPR isolate was done and measured in spectrophotometer.

2.4.2 Seed Inoculation in Bacterial Strain. Three seeds were inoculated with sterilized 0.85% NaCl solution was used as control. Three seeds were inoculated with ~108 CFU/ml of single PGPR isolate from each strain for each pot plant. Three strains were chosen from each pot plant based on high ammonia production reading.

2.4.3 Soybean Growth Analysis. To germinate seeds mixture of different types of soil was made which contained mixture of loam, mud, and sand, 250 grams of soil was used to germinate each PGPR isolated strain. The soil was autoclave for 15 minutes at 121℃ to avoid the contamination. The soybean seeds which were soaked in 0.85% NaCl solution was used as control and the soaked seeds in bacterial strain was labelled as strain selected strain for both isolated strains. The plants were kept 12 hours photoperiod and 12 hours dark period with similar amount of water in room temperature for 4 weeks of time.

2.4.3 Growth Parameter Measurement of Germinated Soyabean Seeds. Two growth parameters were measured during the growth and development of soybean plant. Firstly, the height which was measured in centimeter (cm) and weight was measure in gram (gm) by using digital weight machine. This was done in triplicates as well.

4.0 Findings

4.1 Isolation of PGPR From Pot Plants

In this study we have observe and analyses the two different pot plants which were collected from Sime Darby research facility pot 1 *Rhizophora apiculata* and pot 2 *Rhizophora mucronata.* 40 various isolates have been isolated from rhizospheric region of these two plants soil in which 20 from each plant soil. These isolated strains were analyse based on their morphological and biochemical characteristics.

4.2 Morphological Analysis

4.2.1 Morphological Analysis of *R. apiculata* and *R. mucronate*. Based on morphological appearances there are similarities between some strains which can predict that they belong from similar species. Most of the isolated strain from *R. apiculata* showed a creamy white appearance out of 20 isolates 12 isolates have this appearance, followed by yellow and white. The most formed colony size is small, followed by large and medium in table 1. As for *R. mucronata* the highest color pigment was observed in creamy white out of 20 9 isolates have creamy white followed by white and yellow. The colony size small is the commonly found followed by large and medium.

Table 1. Morphological analysis of *R. apiculate* isolated strains.

Sample name	Color	Opacity	Margin	Size
RAS01	Creamy white	Opaque	Wavy	Large
RAS02	Creamy white	Transparent	Wavy	Small
RAS03	White	Transparent	Even	Small
RAS04	Creamy white	Opaque	Wavy	Large
RAS05	Creamy white	Transparent	Even	Small
RAS06	White	Opaque	Wavy	Large
RAS07	Creamy white	Translucent	Even	Small
RAS08	Creamy white	Translucent	Even	Small
RAS09	Yellow	Translucent	Even	Medium
RAS10	Yellow	Transparent	Curled	Small
RAS11	Creamy white	Transparent	Wavy	Small
RAS12	Creamy white	Transparent	Wavy	Large

RAS13	Creamy white	Transparent	Even	Small
RAS14	Yellow	Translucent	Wavy	Medium
RAS15	Yellow	Translucent	Wavy	Large
RAS16	Creamy white	Translucent	Even	Small
RAS17	White	Transparent	Even	Medium
RAS18	Yellow	Translucent	Wavy	Large
RAS19	Creamy white	Transparent	Wavy	Large
RAS20	Creamy white	Transparent	Wavy	Large

Table 2. Morphological analysis of *R. mucronate* isolated strains.

Sample name	Color	Opacity	Margin	Size
RMS01	White	Translucent	Curled	Medium
RMS02	Creamy white	Opaque	Wavy	Large
RMS03	Creamy white	Transparent	Even	Large
RMS04	Creamy white	Transparent	Curled	Small
RMS05	White	Opaque	Curled	Small
RMS06	Creamy white	Transparent	Wavy	Large
RMS07	Creamy white	Opaque	Even	Large
RMS08	White	Opaque	Curled	Small
RMS09	White	Transparent	Even	Small
RMS10	Creamy white	Transparent	Wavy	Medium
RMS11	Creamy white	Opaque	Even	Large
RMS12	Yellow	Opaque	Curled	Large
RMS13	White	Transparent	Curled	Small
RMS14	Yellow	Opaque	Wavy	Large
RMS15	Yellow	Transparent	Even	Small
RMS16	White	Translucent	Wavy	Small
RMS17	Creamy white	Translucent	Curled	Large
RMS18	Creamy white	Translucent	Even	Small
RMS19	White	Transparent	Even	Small
RMS20	Yellow	Opaque	Even	Medium

4.3 Gram staining

4.3.1 Gram Staining of *R. apiculate* and *R. mucronata* Isolated Strains. The Gram staining analysis was done for *R. apiculata* isolated strains from which 20 isolates 12 isolates showed Gram-positive (purple and blue) and 8 showed Gram-negative (pink and red) results (table 3). The results we obtained from morphological analysis table 1, strains which produced creamy white pigment showed Gram positive result in Gram staining. However, strains with yellow and white showed Gram- negative result. As for *R. mucronata* isolated strains out of 20 isolates 9 isolates showed Gram-positive and 11 showed Gram-

negative results (table 4). The results we obtained from morphological analysis in table 2 strains which produced creamy white pigment showed Gram positive result in biochemical testing. However, strains with yellow and white showed the Gram-negative result. All the microscopic image obtained at 100x magnification from light microscope.

Table 3. Gram staining *R. apiculate* isolated strains, out of 20 isolates 12 showed Gram-positive result and 8 showed Gram-negative result.

Sample Name	Gram Staining
RAS01	Gram-positive
RAS02	Gram-positive
RAS03	Gram-negative
RAS04	Gram-positive
RAS05	Gram-positive
RAS06	Gram-negative
RAS07	Gram-positive
RAS08	Gram-positive
RAS09	Gram-negative
RAS10	Gram-negative
RAS11	Gram-positive
RAS12	Gram-positive
RAS13	Gram-positive
RAS14	Gram-negative
RAS15	Gram-negative
RAS16	Gram-positive
RAS17	Gram-negative
RAS18	Gram-negative
RAS19	Gram-positive
RAS20	Gram-positive

Table 4. Gram staining of *R. mucronate* isolated strain, out of 20 isolates 11 showed Gram-positive result and 9 showed Gram-negative result.

Sample Name	Gram Staining
RMS01	Gram-negative
RMS02	Gram-positive
RMS03	Gram-positive
RMS04	Gram-positive
RMS05	Gram-negative
RMS06	Gram-positive
RMS07	Gram-positive
RMS08	Gram-negative

RMS09	Gram-negative
RMS10	Gram-positive
RMS11	Gram-positive
RMS12	Gram-negative
RMS13	Gram-negative
RMS14	Gram-negative
RMS15	Gram-negative
RMS16	Gram-negative
RMS17	Gram-positive
RMS18	Gram-positive
RMS19	Gram-negative
RMS20	Gram-negative

4.4 PGPR Characteristics

4.4.1 Qualitative Analysis of Ammonia Production in *R. apiculata* and *R. mucronata* isolated strains. The isolated strains which showed color change from brown to yellow were positive result for ammonia production test. However, no color changes considered as negative results for ammonia production test. Isolates from *R. apiculata as well as for R. mucronata out of* 20 isolates 7 showed the color change.

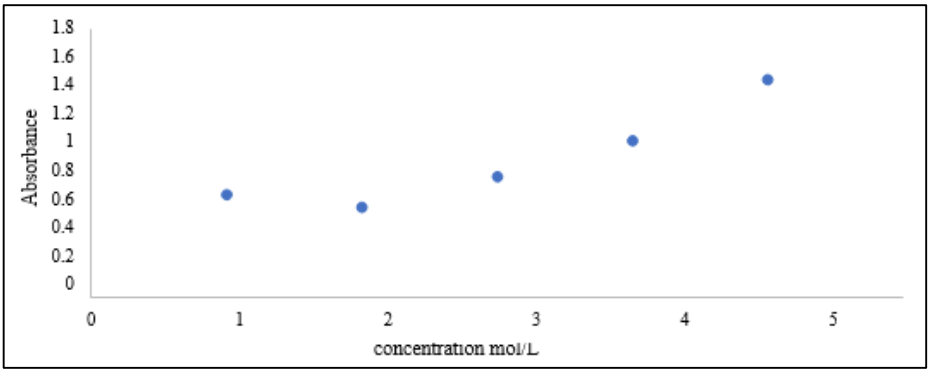

Fig. 1. Standard curve of ammonia production concentration from 1 mol/l to 5 mol/l.

4.4.2 Quantitative Analysis of Ammonia Production in *R. apiculate* and *R. mucronata* Isolated Strains. The positive tested isolates were quantitively analyzed for ammonia production test. In R. *apiculate* the highest ammonia production was observed in RAS01 strain absorbance value of 1.387 which showed the concentration of 4 mol/l in standard

curve chart in and the lowest was produced by RAS15 isolate which is of 0.375 which showed the concentration of less than 1 mol/l of ammonia in figure 1. As for *R. mucronata* isolates strain RMS05 produces the highest absorbance value of 0.58 OD which showed according to standard curve the concentration of 1 mol/l, and the lowest was produced by isolate RMS17 which showed the concentration less than 1 mol/l figure 1.

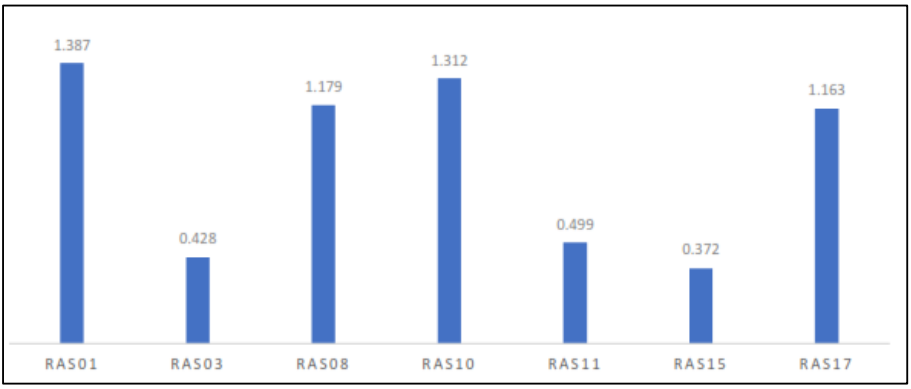

Fig. 2. Graphical representation of quantitative analysis of ammonia production *R. apiculate* isolated strains, the highest absorbance value was observed by RAS01 and lowest was produces by RAS15.

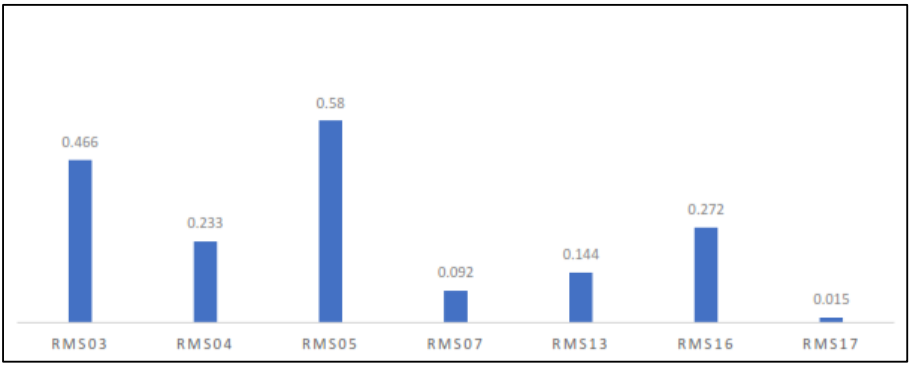

Fig. 3. Graphical representation of quantitative analysis of Ammonia production in *R, mucronata* isolated strains. The highest absorbance value was observed by RMS05 and lowest was produced by RMS17.

4.4.3 Qualitative Analysis of IAA in *R. apiculata* and *R. mucronata* Isolated Strains. The isolated strains which showed color change from yellow to red were positive result for IAA test. However, no color changes considered as negative results for IAA production test. In *R. apiculata* as well as for *R. mucronata* out of 20 isolates 7 showed the color changes and 13 isolates showed no color change.

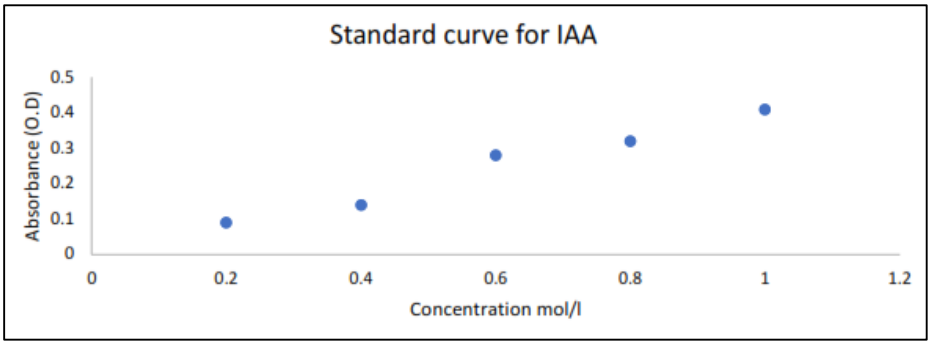

Fig. 4. Standard curve of Indole acetic acid, various concentration of ammonia was prepared to observe the IAA absorbance value.

4.4.4 Quantitative Analysis of IAA in *R. apiculata* and *R. mucronata* Isolated Strains. The positive tested isolates were quantitively analyzed for IAA production test. In *R. apiculate* the highest IAA was observed in RAS10 the absorbance value of 0.379 OD at which can predict the concentration of 0.9 mol/l according to standard curve, the lowest absorbance value was observed in RAS03 isolate which is of 0.172 OD the concentration of less than 0.6 mol/l of IAA in figure 4. In *R. mucronata the highest IAA was produced by* RMS16 absorbance value of 0.291 OD which showed according to standard curve the concentration is above 0.6 mol/l, and the lowest was produced by two isolates produced similar results RMS04 and RMS17 which showed the concentration is above 0.4 mol/l figure 4.

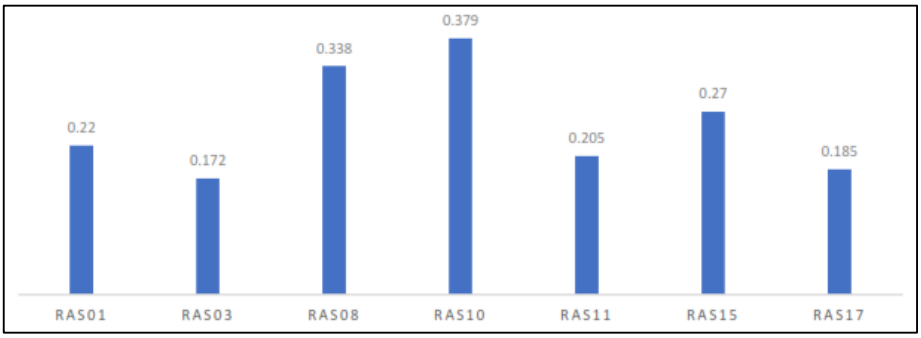

Fig. 5. Graphical representation of quantitative analysis of Indole acetic acid in *R. apiculata* isolated strains, the highest absorbance value observed in RAS10 and lowest was observed in RAS03.

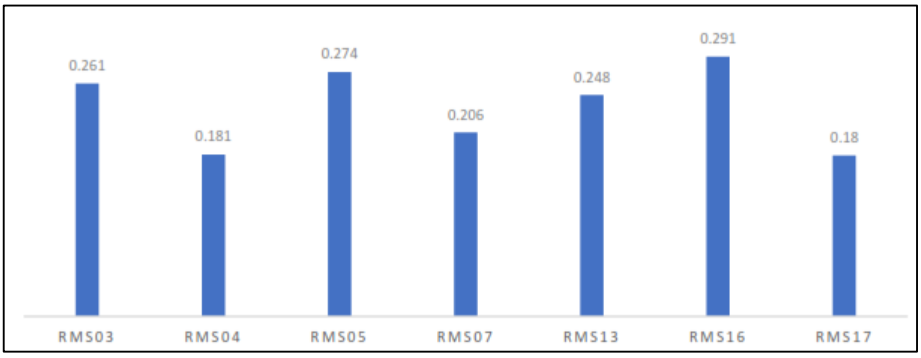

Fig. 6. Graphical representation of quantitative analysis of Indole acetic acid in *R. mucronata* isolated strains. The highest absorbance value was observed by RMS16 and lowest was produced by RMS04 and RMS16.

4.5 Soybean Plant Growth Analysis Under Isolated PGPR Strain from *R. apiculata* and *R. mucronate*.

The soybean plant growth analysis was done after characterizing the isolates based on Ammonia production test. The Three isolates from R. apiculate strains were chosen, RAS01, RAS08 and RAS10 all isolates tested highest height and weight reading for the soyabean plant in comparison with control result. Among the three isolates, RAS10 resulted the highest growth parameter for height and weight table (7 and 8). The isolates from R. mucronate are RMS03, RMS05 andRMS15. Among the observed results from soyabean plants, the lowest reading for growth parameter were observed in RMS03 and RMS16

isolates in comparison with control. However, the highest height and weight reading was observed in RMS05 isolate as compared to control table (9 and 10).

Table 5. Control value for height parameter for Soyabean plant in cm.

Strain	Mean (cm)
Control	55

Table 6. Control value for weight parameter for Soyabean plant in gm.

Strain	Mean (gm)
Control	87.8

Table 7. Height parameter reading for *R. apiculata* strains.

Strains	Mean (cm)
RAS01	61.6
RAS08	65
RAS10	103.5

Table 8. Weight parameter reading for *R. apiculata* strains.

Strains	Mean (gm)
RAS01	90.6
RAS08	93.8
RAS10	130.21

Table 9. Height parameter reading for *R. mucronata* strains.

Strains	Mean (cm)
RMS03	49
RMS05	70
RMS16	51.5

Table 10. Weight parameter reading for *R. mucronata* strains.

Strains	Mean (gm)
RMS03	84.6
RMS05	97.2
RMS16	86.5

5.0 Discussion

In this study, isolated PGPR strains were evaluated for their biofertilizing activity on Soybean plants. The observed results from Morphological and Gram staining characteristics varied among strains for both pot plants, with creamy white pigment and Gram-positive isolates likely belongs to the *Bacillus sp* and strains with yellow or white Gram-negative are likely associated with *Pseudomonas sp* [10-13]. As indicated researcher, *Pseudomonas* and *Bacillus* are most dominant PGPR strains in soil's rhizosphere. The PGPR characteristics as Ammonia production and Indole acetic acid (IAA) tests were conducted as well on the isolated strains. Ammonia production may positively influence plant growth and development [14]. The highest concentration was observed in R. *apiculata isolates*. Notably, *Pseudomonas* strains from *R. mucronata* showed higher ammonia production, while *Bacillus* strains from R. apiculate produced highest ammonia. According to Mishra et al. [14] observation *Bacillus subtilis* strain MA-2 and *Pseudomonas fluorescens* strain MA-4 showed an efficient result of ammonia production.

The Salkowski reagent test revealed varying IAA production levels among the isolated strains, with *R. apiculata* strains producing higher concentrations particularly RAS10 as compared to R. mucronate strains. The highest producing IAA idealy belongs to Pseudomonas species. The IAA biosynthesis in bacteria can either be tryptophan-dependent or independent. Tryptophan-dependent biosynthesis relies on tryptophan as a precursor, using the indole-3-pyruvic acid pathway, and has been observed to enhance root growth [15]. The highest ammonia production was observed in RMS05 from *R. mucronata* and RAS01, RAS08, and RAS10 from *R. apiculata*. Nitrogen availability, facilitated by PGPR bacteria, is crucial for Soybean plant growth. Previous studies also support the positive impact of Pseudomonas and Bacillus species on plant growth, consistent with the findings.

6.0 Conclusion & Recommendation

The isolated PGPR strains from both pot plants are primarily identified as *Bacillus and Pseudomonas sp,* demonstrated the biofertilizing activity which significantly influenced the Soybean plant growth through PGPR traits such as ammonia and IAA production. However, due to certain strains showed higher efficiency in these activities the observation of species-specific variation was observed. The results from this research study emphasize the potential of PGPR in promoting plant growth and development which contributes for sustainable agriculture system. Further investigation can be done to study the root colonization of the isolated strains.

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