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Isolation and identification of nitrogen-fixing bacteria in the long-term fertility experiment in irrigated lowland rice ecosystem

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Abstract Nitrogen-fixing bacteria makes up the large percentage of the soil environment and has a great role for plant growth promotion and anti-fungal activities. In this study, sixteen unknown bacteria were isolated from the control plot of NSIC Rc158 rice variety in the Long-Term Fertility Experiment of Philippine Rice Research Institute in Science City of Muñoz, Nueva Ecija. The identification of the purified isolates was confirmed using direct Polymerase Chain Reaction using 16S rDNA gene marker. Six bacterial isolates were successfully identified and matched the identities of *Bacillus alkalinitrilicus* with 98% identity, *Pseudomonas nitroreducens* with 100% identity, *Streptomyces corchorusii* with 100% identity, *Actinomadura* sp. *with 99% identity, Sediminicoccus rosea* with 100% identity and *Bacillus megaterium* with 99% identity. Some of these bacteria were known to be a biocontrol agent, potential biofertilizer and also a good bio-remediator in the field of agriculture. Identification of all the bacteria presented on different rice varieties with different fertilizer input using *nifH* gene marker were recommended in this study.

Keywords: Long-Term Fertility Experiment (LTFE), Nitrogen-fixing bacteria, Rice, 16S rDNA analysis

Introduction

More than a billion people depend on rice cultivation for their livelihoods; hence, it is considered as one the most important crops in the world (Ladha and Reddy, 2002). Similarly, more than 3.5 billion people depend on rice as good source of their daily calories (Ahn *et al*., 2012). According to Ricepedia (2017), Philippines is considered as the world's eighth-largest rice-producer with 5.4 million hectares of arable lands. Harvested area

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for rice has expanded from nearly 3.8 million hectares to about 4.4 million hectares (from 1995 to 2010). However, areas dedicated for rice harvesting in the country are still very small compared with other major rice-producing countries in Asia.

Increase in population lead to an increase in demand for rice which creates the need to improve rice productivity. Fertilizer input and pesticides are one of the most important factors in obtaining high yield of rice which may cause environmental pollution and negatively influence human health. Nitrogen, phosphorus, and potassium play an important role in the growth and yield of crops (Fageria *et al.,* 2011). Among the three major components of chemical fertilizers, nitrogen is the most limiting nutrient in rice production (Fageria *et al.,* 2003).

Rice requires one (1) kg of nitrogen to produce 15–20 kg of grain (Ladha and Reddy, 2000). In the tropics, lowland rice yields 2–3.5 Mg ha−1 using naturally available N derived from biological nitrogen fixation (BNF) by free-living and plant-associated diazotrophs and from mineralization of soil N (Fischer, 2000). Under specific favorable conditions, bacteria that are free-living in soil (*e.g. Azospirillum* and *Azotobacter*) may fix significant amounts of nitrogen (0 to 60 kg N ha^{-1} $year^{-1}$) that would benefit the plants **(**Burgmann *et al.,* 2004).

Biological nitrogen fixation (BNF) is the process of reduction of atmospheric nitrogen to ammonia carried out by a large number of species of free-living and symbiotic microbes called diazotrophs. BNF presents an inexpensive and environmentally sound, sustainable approach to crop production and considered as one of the most important Plant Growth Promotion (PGP) scenarios (De Bruijin, 2014). Aside from nitrogen fixation, diazotrophs can affect plant growth directly by the synthesis of phytohormones, inhibition of plant ethylene synthesis, improved nutrient uptake, enhanced stress resistance, solubilization of inorganic phosphate and mineralization of organic phosphate (Dobbelaere *et al*., 2010). The soil environment is likely the most complex biological community with extremely diverse microorganisms that contributes to soil fertility which are essential to the sustainable function of natural and managed ecosystems (Barrios, 2007).

Hence, identifying beneficial microorganisms like nitrogen-fixers can contribute to the knowledge of farmers regarding soil fertility and for them to apply this knowledge on managing their farms. This study aimed to isolate nitrogen-fixing bacteria in the long-fertility experiment in irrigated lowland rice ecosystem and identify it by morphological and molecular approach.

Materials and methods

Soil sample collection

Soil collection was conducted at the NSIC Rc158 variety control set-up in the Long-Term Fertility Experiment of Philippine Rice Research Institute, Science City of Muñoz, Nueva Ecija. Soil samples were collected at three sampling equidistant points in each plot with 20 cm depth during vegetative and reproductive stages of the rice. Samples were placed in sterilized polypropylene plastic bags with labels and brought to the laboratory for processing.

Media preparation

In 1000 ml distilled water, 39.1 grams of Jensen's medium (Sucrose 20.0g, Dipotassium phosphate 1.00g, Magnesium sulphate 0.50g, Sodium chloride 0.1g, Ferrous sulphate 0.1g, Sodium molybdate 0.005g, Calcium carbonate 2g and Agar 15g) were suspended and heated to dissolve all the particles completely. The medium was autoclaved at 121℃ in 15 psi for 20 minutes, cooled down and poured into sterilized petri dishes. This medium was selective for nitrogen-fixing bacteria.

Isolation and purification

Soil samples were serially diluted by suspending 1g of soil sample in 9ml of sterilized distilled water and shaken thoroughly using vortex mixer. Using a sterile pipette, 1ml of soil solution was transferred in the first tube of the set labeled as 10^{-1}. The contents were mixed by gently swirling the tube upside down. Then, 1ml of the solution was transferred in the tube labeled 10^{-2}. The procedure was repeated with the remaining tube labelled as 10^{-3}. Sample with 10^{-3} dilution factor was used in the spread plating to obtain bacterial isolates. A 0.1 ml of soil solution was aseptically pipetted out. Using the sterile L-shaped glass spreader, the sample was spread out evenly over the surface of agar by carefully rotating the petri dish. Plates were incubated at room temperature (28-30^{o}C) for 24 hrs. For the isolation of pure culture, colonies that are well-separated from neighboring colonies were picked and selected to perform streak-plate method in duplicate.

Molecular identification of nitrogen-fixing bacteria

Direct polymerase chain reaction

Bacterial amplification was taken directly from the pure cultures of the isolated bacterial samples. Sterilized toothpick was used in transferring a

bacterial culture directly into the prepared PCR reaction mixture. The near full-length 16s gene region was amplified using the 16SF (5'-CCAGCAGCCGCGGTAATACG-3') and 16SR (5'-ATCGGCTTACTTTGTTACGACTCC-3') primer pair. Reactions were carried out in a 0.2 ml per tubes of 45 µl containing: 5 µl of 10X PCR buffer, 1.25 µl of dNTP mix, 2.5 ul of forward primer (16SF), 2.5 ul of reverse primer (16SR), 0.2 µl of Taq polymerase and 33.5 µl of sterilized distilled water, and a small portion of the bacterial culture. The PCR was performed using Thermal Cycler (Applied Biosystem®2720) and run with the following PCR profile: 2 min at 95℃ (initial denaturation) followed by the 35 cycles of 20 sec at 95℃ (denaturation), 30 sec at 52℃ (annealing), 1 min at 72℃ (extension) and 5 minutes at 72℃ (final extension) and final step at 10℃.

Gel electrophoresis of amplification products

PCR amplification products were resolved in horizontal gel electrophoresis to check for quality and molecular weight. A mixture of 3 µl amplification product and 4 µl loading dye was loaded into 1.5% agars gel containing 2 µl gel red prepared from 1X Tries-acetate EDTA (1X TAE) with nucleic acid dye buffer along with standard DNA ladder. Electrophoresis was carried out at 100 V for 30 min. The amplified products were checked using gel documentation system (Endure™ GDS Gel Documentation System).

Sequence and BLAST Analysis

Samples with amplification were stored in the microcentrifuge tubes and sealed with parafilm and sent to Apical Scientific Sequencing in Malaysia for PCR purification and sequencing procedure**.** After sequencing, the sequences were viewed using BioEdit. BLAST was performed to confirm the identity of the isolates based on nucleotide sequence similarity available from NCBI (Altschul *et al*., 1997). Phylogenetic analysis was done using default parameters of the MEGA X (Kumar *et al*., 2018; Tamura and Nei, 1993). using the 16S rDNA region of the isoalates and related sequences used in this study. Phylogenetic tree was constructed by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model (Kumar *et al*., 2018; Stecher *et al*., 2020).

Morphological Identification

Gram staining

The bacterial samples were transferred using a sterile inoculating loop in a glass slide containing a drop of sterile water. Microbial isolates were smeared

and was heated to fix the cells. The sample was gently flooded with crystal violet and allowed to stand for one minute and was gently rinse with water and blot dried. Then, sample was gently flooded with Gram's iodine and allowed to stand for one minute and was gently rinsed with water and blot dried. Afterwards, it was decolorized using Gram's alcohol for 15-30 seconds and was rinsed with water and blot dried. Then, sample was flooded with Safranin O and let stand for 30 seconds to 1 minute and rinsed with sterile water. Each sample was observed under the microscope using oil immersion objective.

Results

Isolation of bacteria on the rhizosphere

A total of 16 potential nitrogen-fixing bacteria were isolated from the plot of NSIC Rc158 rice variety in the Long-Term Fertility Experiment (LTFE) of Philippine Rice Research Institute, Maligaya, Science City of Muñoz, Nueva Ecija (Figure 1).

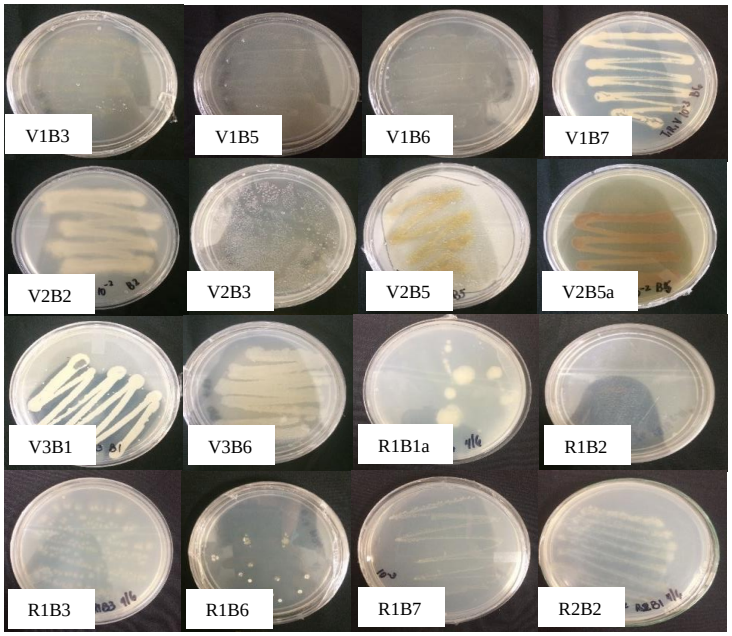

Figure 1. Sixteen unknown bacteria isolated from the Long-Term Fertility Experiment (LTFE) in Philippine Rice Research Institute, Science City of Muñoz, Nueva Ecija

Molecular identification of bacterial isolates

Six unknown isolates were successfully identified using 16S rDNA analysis: R1B1a matched the identity of *Bacillus alkalinitrilicus* (accession number NR044204.1) with 98% maximum identity based on 339-bp length; R1B2 identified as *Pseudomonas nitroreducens* (accession number CP049140) with 100% maximum identity based 740-bp length; R1B3 as *Streptomyces corchorusii* (accession number KY412832.1 with 100% maximum identity based on 417-bp length; R1B6 as *Actinomadura sp*. (accession number KY458126.1) with 99% maximum identity based 290-bp length; R1B7 as *Sediminicoccus rosea* (accession number KY029040.1) with 100% maximum identity based on the 414-bp length and R2B2 as *Bacillus megaterium* (accession number KP886467.1) with 99% maximum identity based on 662-bp length (Table 1). Figure 2 shows the gel image of the amplified products (band ~500 bp) of the selected isolates. Figures 3-8 show the phylogenetic tree of each isolate.

Table 1. Identities of the selected bacterial isolates based on 16S rDNA marker gene

SAMPLE ID	ACCESSION NAME	ACCESSION NUMBER	QUERY LENGTH	% IDENTITY
R1B1a	*B. alkalinitrilicus*	NR044204.1	339	98%
R1B2	*P. nitroreducens*	KY849350.1	740	100%
R1B3	*S. corchorusii*	KY412832.1	417	100%
R1B6	*Actinomadura sp.*	KY458126.1	290	99%
R1B7	*S. rosea*	KY029040.1	414	100%
R2B2	*B. megaterium*	KP886467.1	662	99%

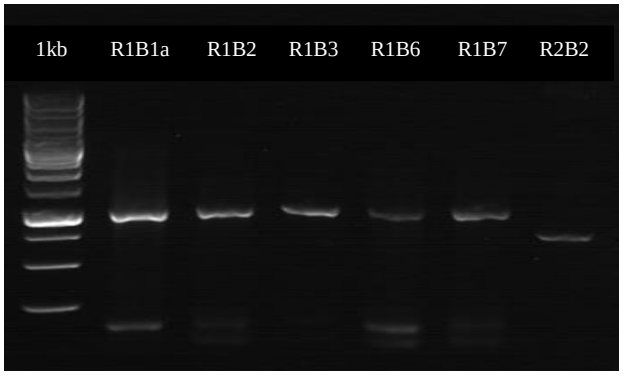

Figure 2. Gel image of the PCR amplified products of the selected isolates (band ~500 bp)

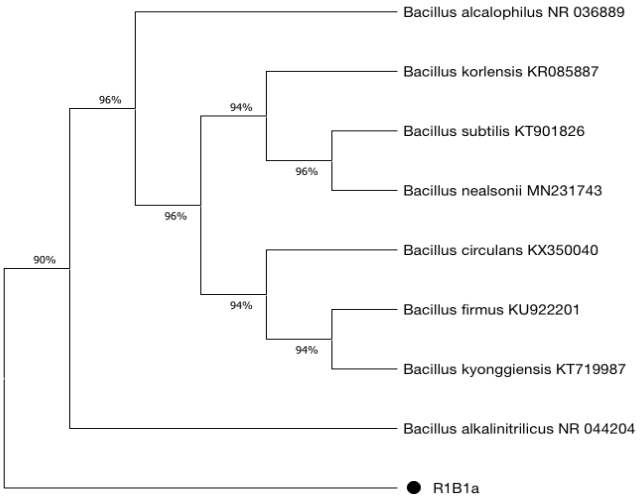

Figure 3. Phylogenetic tree based on 16S rRNA sequence of R1B1a including the eight related sequences extracted from NCBI. The R1B1a isolate and its phylogenetic position was shown in black dot. The phylogeny involved 9 related sequences; the accession numbers of the reference strains are shown after the name of the strains

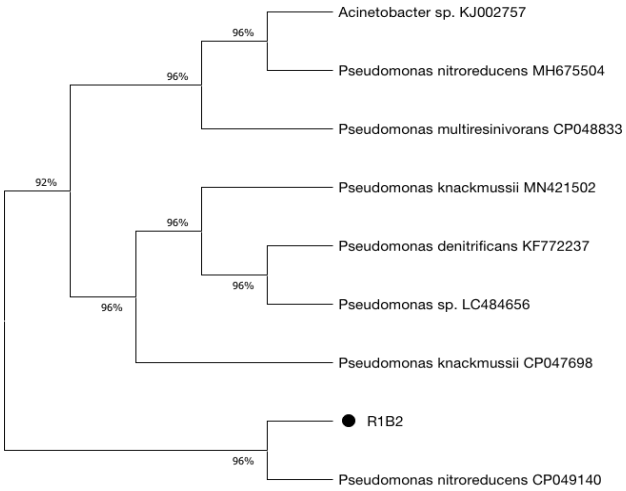

Figure 4. Phylogenetic tree based on 16S rRNA sequence of R1B2 including the eight related sequences extracted from NCBI. The R1B2 isolate and its phylogenetic position was shown in black dot. The phylogeny involved 9 related sequences; the accession numbers of the reference strains are shown after the name of the strains

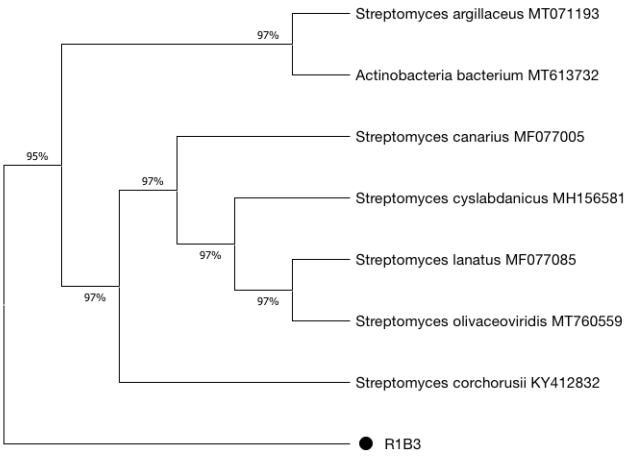

Figure 5. Phylogenetic tree based on 16S rRNA sequence of R1B3 including the seven related sequences extracted from NCBI. The R1B3 isolate and its phylogenetic position was shown in black dot. The phylogeny involved 8 related sequences; the accession numbers of the reference strains are shown after the name of the strains

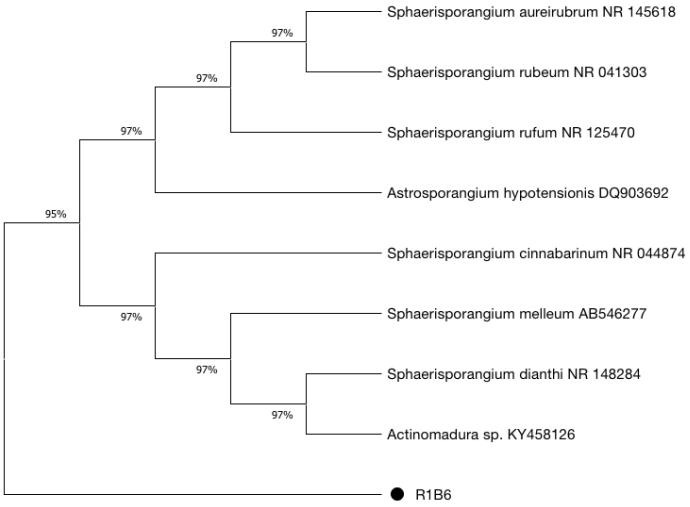

Figure 6. Phylogenetic tree based on 16S rRNA sequence of R1B6 including the eight related sequences extracted from NCBI. The R1B6 isolate and its phylogenetic position was shown in black dot. The phylogeny involved 9 related sequences; the accession numbers of the reference strains are shown after the name of the strains

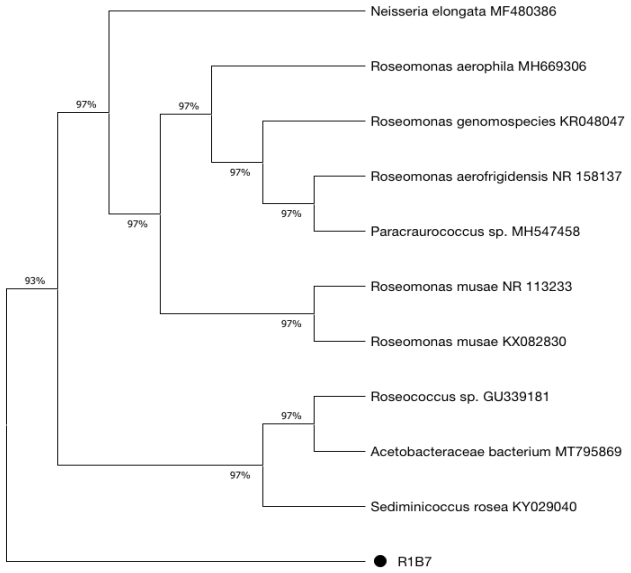

Figure 7. Phylogenetic tree based on 16S rRNA sequence of R1B3 including the ten related sequences extracted from NCBI. The R1B3 isolate and its phylogenetic position was shown in black dot. The phylogeny involved 11 related sequences; the accession numbers of the reference strains are shown after the name of the strains

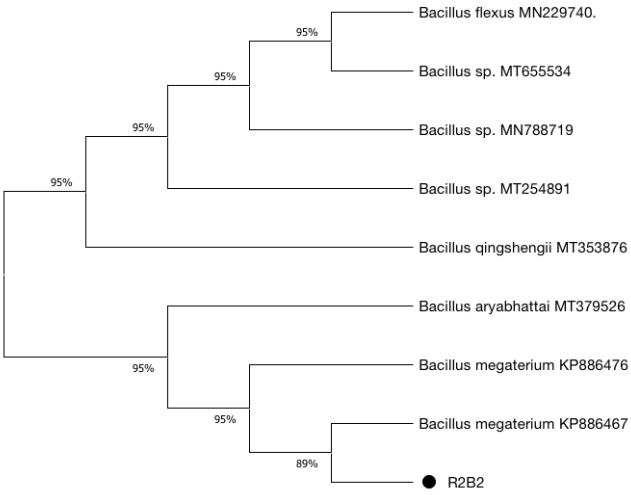

Figure 8. Phylogenetic tree based on 16S rRNA sequence of R1B3 including the eight related sequences extracted from NCBI. The R1B3 isolate and its phylogenetic position was shown in black dot. The phylogeny involved 9 related sequences; the accession numbers of the reference strains are shown after the name of the strains

Morphological identification of bacterial isolates

After the molecular identification of the six isolates, it was subjected to Gram staining to know their morphological characteristics as shown in Table 2 and Figure 3. Based on the results, *B. alkalinitrilicus*, *S. corchorussii*, *Actinomadura* sp. and *B. megaterium* were gram positive as shown by the blue stain retained on their cell wall. On the other hand, isolates *P. nitroreducens* and *S. rosea* were Gram-negative. Isolates *B. alkalinitricus* and *B. megaterium* are rod in shape while *S. corchorussii* and *Actinomadura* sp. are filamentous.

Table 2. Morphological characteristics of the selected isolates

IDENTITY	CELL SHAPE	GRAM +/-
B. alkalinitricus	rod	+
P. nitroreducens	rod	-
S. corchorussii	filamentous	+
***Actinomadura* sp.**	filamentous	+
S. rosea	coccus	-
B. megaterium	rod	+

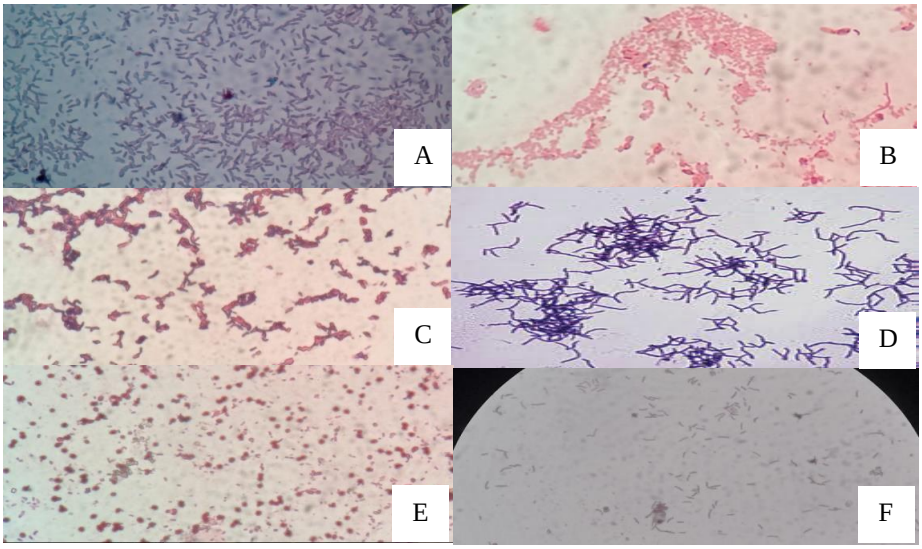

Figure 3. Morphological characteristics of the six bacterial isolates. (A) *B. alkalinitricus*; (B) *P. nitroreducens*; (C) *S. corchorussii*; (D) *Actinomadura* sp.; (E) *S. rosea* and (F) *B. megaterium*

Discussion

B. alkalinitrilicus (R1B1a) is a gram-positive fat rods with pointed edges highly motile with peretrichous flagella and forming round subterminal endospores and strictly aerobic (Sorokin *et al.*, 2008). This species of bacteria

was reported as a bioremediator (Sharma *et al.,* 2014) and was known to be a plant growth-promoting bacterium which exert beneficial effect on plants through fixing atmospheric nitrogen. It was also reported that this species showed high potential for biofertilization contributing to better yield of diverse field and horticultural crops (Baset *et al.,* 2017).

Isolate *P. nitroreducens* (R1B2) cell on the other hand, is rod-shaped, Gram-negative and motile. This species of bacteria has potential plant benefits and environmentally useful characteristics. It was also the first strain to be isolated in 1974 that could degrade chlorinated aromatic hydrocarbons (Miyazaki *et al.,* 2014). Meanwhile, *S. corchorusii* (R1B3) was a Gram-positive filamentous bacterium that produces well-developed vegetative hyphae (between 0.5-2.0 µm in diameter) with branches (Chater, 1984). This species was reported to have a plant growth-promoting activity such as nitrogen fixation and serves as a biocontrol agent against six major rice fungal pathogens (Tamreihao *et al.,* 2016).

Isolate *Actinomadura* sp. (R1B6) is an aerobic, Gram-positive, catalase-positive, non-acid fast, and non-motile actinomycete. This actinomycete peformed the following plant growth-promoting activities: nitrogen fixation, biocontrol activities and siderophores. Siderophore-producing bacteria have the ability to make the plants resistant to pathogens (Khamna *et al.,* 2009; Dhakal *et al.,* 2017).

On the other hand, *S. rosea* (R1B7) species does not have literatures that can confirm its morphological characteristics and its potential benefit that may contribute to the environment. Lastly, *B. megaterium* (R2B2) cells was rod-like, Gram-positive and mainly aerobic spore-forming bacterium found in widely diverse habitats. The cells often occur in pairs and chains (De Vos *et al.,* 2011). This species also reported to be effective on enhancing nitrogen content which helps in plant growth promotion as reported by Hesham and El-Komy, (2005).

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