In Vitro Enhancement of Rice Seedling by Phosphate Solubilizing Bacteria from the Rhizosphere of Grass

Sandar Moe
Pathein University
sandarmoe1986@gmail.com

Yamin Kyaw
University of Mandalay

Wai Zin Min
Hinthada University

Abstract

The aim of this research paper is the isolation of phosphate solubilizing bacteria from rhizosphere soil and hence the production of biofertilizer from it. A total of seven bacteria were isolated from rhizosphere of grass naturally growing near the Recreation Centre in the Pathein University Campus, Ayeyarwady Region during July to November, 2019. Based on results of enumeration and solubilization index, only four isolated bacteria, PSBGS-1 (T1), PSBGR-2 (T2), PSBGR-4 (T3) and PSBGR-6 (T4) were selected as inoculants to test the effect of these bacteria on the growth of rice. Most biochemical test results showed isolated bacteria may belong to the genera of Pseudomonas and Bacillus. In this study, shoot length of all treatments are significantly ($p<0.05$) longer than over control except at 5^{th} and 7^{th} day and then root length were also significant over control. So, they can be used as inoculums for the production of phosphate based biofertilizer.

Keywords*: Rice, phosphate solibilizing bacteria, grass*

1. Introduction

Phosphorus (P) is one of the major essential macro nutrients for plants. However, a greater part of soil phosphorous, approximately 95-99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants. Phosphate solubilizing bacteria (PSB) are capable of solubilizing different forms of inorganic phosphates by acidification, chelation and exchange reactions in the periplasm, which
function as an indicator for general isolation and selection procedure of phosphate solubilizers [1]. The challenges for the next decades include understanding the behavior of microbes in their natural and often complex habitats, such as the rhizosphere. Hence, searching alternative strategies to improve soil health and crop yield without causing damage to the soil and environment is a focus of researchers for a number of years. Biofertilizers are, therefore gaining importance as they are eco-friendly, nonhazardous and non-toxic [2].

Phosphate solubilizing bacteria (PSB) can grow in media containing tri-calcium phosphate as the sole phosphorus source, and can solubilize, assimilate and release phosphorus in higher amounts. This reaction, manifested as a halo or a clear zone on the plate, is used for assessing the phosphate solubilizing activity of these bacteria [9].

Bacterial isolates were identified as PSB base on their ability to solubilize tricalcium phosphate $Ca_3(PO_4)_2$ by the formation of visible dissolution halos on Pikovskaya agar. The use of phosphate solubilizing bacteria as inoculants simul-taneously increases phosphorus uptake by the plant and crop yield. Strains from the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Azospirillum*, *Enterobacter*, *Erwinia*, *Serratia*, *Arthrobacter* and *Flavobacterium* are among the most powerful phosphate solubilizers [3]. Rice is also grown from sea level to 3000 m and in both temperate and tropical climates. Plant-growth promoting rhizobacteria (PGPR) can function as potential bio-inoculants for promotion of growth and development of plants especially rice. Rice (Oryza sativa) is the stable food for half of the world's population especially in oriental countries [4].

Prolonged use of chemical fertilizers has resulted in a number of negative side effects on the environmen. Biofertilizers are gaining an importance in use because of the proper maintenance of soil health, minimize environmental pollutions and cut down the use of chemicals. Several soil bacteria, particularly those belonging to the genera *Pseudomonas* and *Bacillus* were reported to be involved in solubilizing phosphorus [5].

Phosphorus solubilizing bacteria (PSB) play an important role in phosphorus (P) nutrition by enhancing plant P availability. These PSB have been shown to improve growth and yield of rice [6]. Current study would loop out further avenues for researchers interested to commercially produce the PSB based bio-fertilizers to be effective over a wide range of crops.

2. Materials and Methods

2.1. Sampling Site and Soil Sample Collection

Rhizosphere of grass was collected naturally growing near the Recreation Centre in the Pathein University Campus (16º 48' 11" N and 94º 45' 19" E) .The portions of roots were taken from 5 to 15 cm below the stem base. Soil temperature, pH, weather and time of collection were also recorded.

2.2 Isolation of PSB from Soil sample

PSB were isolated from each sample by serial dilution and spread plate method. One gram of rhizospheric soil sample was weight and put into test tube with 10 mL of sterilized distilled water. The soil sample was vigorously agitated and then the supernatant

was diluted 10^{-1}-10^{-10}. 20 mL of each dilution was inoculated on to Pikovskaya's medium (PVK) (Himedia, India) containing insoluble tricalcium phosphate and incubated at 27°C-30°C for 7 days. Clear zone forming colonies were selected and restreaked on to PVK medium for pure isolation.

2.3. Measuring of Clear Zone on PVK medium

Isolated bacteria were inoculated in PVK broth at 27°C-30°C. Then a 10µL top of bacterial suspension of each strain was inoculated and incubated at 27°C-30°C for 7 days. After this clear zone and colony diameter were measured.

2.4. Biochemical Tests of Isolated Bacteria

Base on the results of enumeration and solubilization index, only four isolated bacteria, PSBGS-1 (T1), PSBGR-2 (T2), PSBGR-4 (T3) and PSBGR-6 (T4) were selected using KB003 Hi 25 TM identification Kit (Himedia, India). Isolated bacteria from pure culture were added to the wells of kit following the instructions supplied by the manufacturer. Identification was followed after Bergey's Manual of Determinative Bacteriology [7]. Gram's staining was used to identify the bacteria species. After staining, these were examined under light microscope (×1000).

2.5. Inoculum Preparation

Isolated bacteria were grown in Pikovskaya's (PVK) media. Pure culture of bacteria were grown in 10 ml peptone water for 24 hours at 37ºC and final concentration of inoculums were made to 10^6 CFUmL$^{-1.}$

2.5.1. Sterilization of Seeds and Inoculation of Seeds

Rice seeds were sterilized with 0.1 % NaOCl for 2 to 3 minutes. These seeds were washed four times with sterilized distilled water. Seeds were immersed in each bacterial inoculum (10^6 CFU mL^{-1}) and 3mLof insoluble phosphate (0.5%) was added into each treatment for 3 hrs. Control seeds were only immersed in diluted peptone water. Inoculated seeds were put in sterilized Petri dishes (30 seeds per dishes) containing filter paper. Each treatment with five replicate were carried out to test the effect of isolated bacteria.

2.5.2. Germination Parameters

The plate were kept in dark for 5 days and subsequently subjected to light condition for another 5 days under room temperature. Microbe solution was added during the exposure on light. Measurement of root and shoot lengths were conducted 3 days after sowing (DAS) and continuously taken until 10 DAS. The seed germination, length and weight of root and shoot were also measured. Percentage of seed germination was calculated by the following equation [8];

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \text{x } 100$$

2.6. Statistical analysis

Data of the experiment were subjected to statistical analysis using IBM-SPSS software (version 25). The differences between the treatment and control means were determine by using One-way ANOVA with LSD, post-hoc test.

3. Results

Total of seven strains were isolated from the grass with Pikovskaya's medium (PVK). All isolated strains showed clear zone on PVK medium. These isolated strains were designated as PSBGS-1, PSBGR-2 to 7 (phosphate solubilizing bacteria from roots and soil).

3.1. Colony Morphology and Cell Morphology of PSB from Grass

Seven strains of PSB were isolated by serial dilution method from grass on PVK medium. After incubation of four days at 27°C-30°C, formation of clear zone were observed. The colony feature of PSBGS-1 and PSBGR-2 were yellow colors and PSBGR-3 to 7 were creamy white color and circular with entire edge. In the elevation of PSBGS-1 and PSBGR-2 are convex and other five strains are flat. The diameters of colony size were 10 to 24 mm on the PVK medium while the diameters of clear zone were range from 18 to 30 mm. Among them, PSBGR-4 is the largest diameter of clear zone. Cell morphology of all isolated bacteria were rod, singly pair and motile and cell size range from 2.5 to 10 µm were observed.

3.2 Biochemical Tests of Isolated Bacteria

All strain were Lysine, Ornithine utilization, Glucose, Nitrate reduction, Voges Proskauer's, Glucose and Lactose positive, and H_2S production, Esculin hydrolysis, Indole, Rhamnose, Melibiose negative. Based on the biochemical test characteristics, phosphate-solubilizing bacterial strains were identified as follows: PSBGS-1 (T1) and PSBGR-2 (T2) were *Pseudomonas* spp., PSBGR-4 (T3) and PSBGR-6 (T4) were *Bacillus* spp., respectively. And then, gram negative of isolated bacteria were T1 and T2 and another two selected strains were gram positive.

3.3. Plant Growth Parameter and Germination Rate

The percentage of germinated seeds increased over their respective controls (Table 1). Shoot length of T1, T2 and T3 were significantly ($p<0.05$) increased over control whereas root length also significantly T2 and T3 at 3rd day (Table 2 and 3). For fresh shoot/root weight and dry shoot/root weight were measured 5 days

intervals. Among of these treatments, T2 showed the highest growth promoting activity in all the parameters except dry shoot weight and dry root weight (Figure 1 and 2).

Table 1. Effect of isolated bacteria on the germination rate of rice

Treatment	Germination rate (Mean±SD)	Percentage of germinated seeds (%)
Ctrl	28.96±0.91	96.53
T1	29.56±0.46	98.53
T2	29.96±0.09	99.87
T3	29.88±0.18	99.60
T4	29.72±0.33	99.07

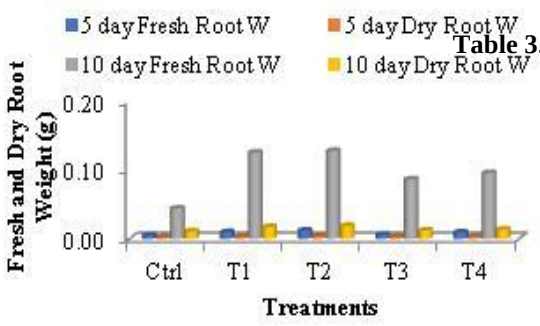

Table 3. Comparison of

Figure 1. Mean fresh and dry shoot weight of rice seedling

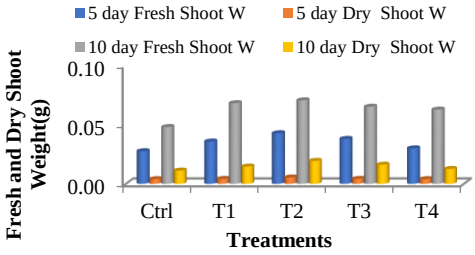

Figure 2. Mean fresh and dry root weight of rice seedling

Table 2. Comparison of mean shoot length of rice seedling

Treatments	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
Ctrl	0.89±0.09^a	1.59±0.27^a	2.34±0.37^a	2.95±0.32^a	4.19±0.50^a	4.29±0.44^a	4.20±0.50^a	4.53±0.66^a
T1	1.08±0.16^b	1.72±0.18^a	2.52±0.11^a	3.66±0.56^b	4.60±0.25^a	4.83±0.18^b	5.08±0.31^b	5.69±0.29^b
T2	1.17±0.07^b	2.11±0.28^b	2.76±0.28^a	3.96±0.20^b	4.71±0.21^b	4.93±0.18^b	5.25±0.23^b	5.83±0.44^b
T3	1.13±0.15^b	1.97±0.13^b	2.61±0.18^a	3.86±0.20^b	4.66±0.21^a	4.91±0.19^b	5.19±0.21^b	5.63±0.32^b
T4	1.05±0.16^a	1.71±0.31^a	2.52±0.70^a	3.60±0.19^b	4.51±0.31^a	4.79±0.39^b	5.17±0.27^b	5.65±0.40^b

Table 3. Comparison of mean root length of rice seedling

Treatments	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
Ctrl	2.83±0.59^a	4.07±0.25^a	5.04±0.42^a	5.21±0.65^a	5.54±0.46^a	5.65±0.65^a	5.76±0.36^a	6.08±0.61^a
T1	3.42±0.56^a	4.63±0.31^a	5.17±0.55^a	5.32±0.69^a	5.64±0.25^a	6.43±0.81^a	6.83±0.61^b	6.97±0.74^a
T2	3.84±0.67^b	5.33±0.77^b	5.98±0.34^b	6.35±0.42^b	6.69±0.51^b	7.08±0.76^b	7.50±0.80^b	8.01±0.80^b
T3	3.71±0.32^b	4.64±0.62^a	5.58±0.46^a	5.75±0.25^a	5.90±0.27^a	6.09±0.78^a	6.55±0.35^b	6.76±0.22^a
T4	3.53±0.70^a	4.22±0.71^a	5.20±0.56^a	5.47±0.47^a	5.94±0.60^a	6.33±0.44^a	6.56±0.56^b	7.03±1.19^a

Ctrl= Control, T=Treatment
Means followed by a common letter in the same column are not significantly different at 5% level by LSD

4. Discussion

In the present research, seven strains of phosphate solubilizing bacteria were isolated from the rhizosphere of grass. Appearance of clear zone around the microbial colonies in media was an indication for the present of PSB and hence these colonies were isolated and aseptically transferred to PVK medium to produce pure culture. The phosphobacteria were identified by noting the solubilizing zone formed around the bacterial colony [9].

Guar et al. [10] studied the PSBs with their morphological, cultural, physiological and biochemical characteristics using the Bergey's Manual of Determinative Bacteriology and identified the organism as *Bacillus* spp.

Meyer *et al*. [11] stated that genus Pseudomonas characterized as rod-shaped, gram negative, one or more polar flagella, providing motility, aerobic or some are facultative anaerobes, non-spore forming and positive catalase test.

In this work, the morphological and biochemical characters of T1 and T2 were found to be Gram negative with rod shaped and motile characteristics. The organism showed positive results for oxidase, citrate utilization, catalase test, voges proskauer's and glucose. Endospore not produces, and was negative for urease, H_2S production. The comparison of characteristic of T1 and T2 with the above, they may be *Pseudomonas* sp.

Sinha and Paul [12] described that PSB strain was found to be Gram positive, rod shaped, and motile, which demonstrates physiological properties primarily indicative of the genus *Bacillus.*

In this investigation, the cell characters and staining reactions of T3 and T4 are nearly the same with the above work. Cells were Gram positive and motile in nature. They were positive to catalase, citrate and voges proskauer's and were negative for indole production, oxidase, H_2S production. They are produce spores. So, they may be possible of genus *Bacillus.*

10^6 CFU mL^{-1} is the optimal concentration for many plant species [13]. Kyaw Myo Naing and Thant Zin [14] also used 106 CFU mL-1 of bacterial inoculum and good effect on rice growth was observed.

In this work, 10^6 CFU mL^{-1} of bacterial concentration was used as inoculum. The germination rate of isolated bacteria treated seedlings excelled over untreated one. Isolate bacteria PSBGR-2 (T2) is the best germination rate and then PSBGS-1 (T1) lower than other treatments but more than control on germination rate. All the treatment showed significantly results over the non-inoculated controls. Shoot length of all treatments are significantly ($p<0.05$) longer than over control except at 5^{th} day and 7^{th} days and then root length were also significant over control except at 3 days and 6 days.

5. Conclusion

The research of this study have shown that the PSB were isolated from the rhizosphere soils of grass in Ayeyarwady Region and colony morphology, cell morphology, and biochemical tests were studied *in vitro*. All isolated PSB can solubilize insoluble phosphate to soluble phosphate for plant growth. The isolated bacteria from grass (*Kyllinga bulbosa* B.) into paddy plant showed the enhancement of germination, shoot and root length. Isolated PSB have been showed positive effects on growth parameter of rice seedling. So, they can be used as inoculums for the production of phosphate based biofertilizer.

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