Isolation of Phosphate Solubilizing Bacteria from Four Different Soil Samples (Pathein Township) and their Effect on *Vigna mungo* L. (Black Gram)

Myint Myint Than[1], Zar Zar Yin[2]

Abstract

Four soil samples were collected from agricultural field at Taung-Yar-Kone village of Pathein Township, Ayeyarwady Region. These soil samples were cultured on Pikovskaya (PVK) medium and a total of 19 bacterial strains were obtained. These bacteria were designated as MMT 1-19. In the colony, the isolated bacteria were small, medium and large in size and the color were white, pale yellow, yellow, cream and brown. The margin of colonies were entire and the elevations were raised and flat. Five bacteria were gram-positive and fourteen strains were gram-negative. Out of 19 bacterial isolates, six strains were found to be potent phosphate solubilizers showing clear zone around their colonies. In the effect of phosphate solubilizing bacteria on *Vigna mungo* L. (Black gram), six isolates (MMT-3, MMT-8, MMT-12, MMT-13, MMT-14 and MMT-16) were screened by seed germination, survival and plant height. The tallest plants were obtained by using Treatment 1 (MMT-3) at 7 days (9.54 cm) and 14 days (19.52 cm). The shortest plants were observed in control at 7 days (5.63 cm) and 14 days (12.02 cm). The value of seed germination percentage and survival were the maximum percentage (92%) in Treatment 1 (MMT-3) followed by 88% in Treatment 2 (MMT-8) both after 7 days and 14 days.

Keywords: Phosphate Solubilizing Bacteria**,** Biofertilizer

Introduction

Phosphate Solubilizing Bacteria (PSB) are the bacteria that possess the capability to change the insoluble form of phsophorus into soluble one. Phosphorus is one of the most essential elements for plant growth only to nitrogen in requirement for plants. Phosphorus plays a significant role in physiological and biochemical plant activities. But, due to different chemical reactions, there is limited availability of this nutrient for plants especially in arid and semi-arid soils. Most of the essential plant nutrients remain in insoluble form in soil (Abdalla 1994).

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available for root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition (Ahmad Ali Khan *et al.*, 2009). 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 (Kannapiran and Sri Ramkumar, 2011). Phosphorous plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant. It helps to survive winter rigors and also contributes to disease resistance in some plants (Amit Sagervanshi *et al.*, 2012). P availability is low in soils because of its fixation as insoluble phosphates of iron, aluminium and calcium. Since deficiency of P is the most important chemical factor restricting plant growth, chemical phosphatic fertilizers are widely used to achieve optimum yields. Soluble forms of P fertilizer used are easily precipitated as insoluble forms, and this leads to excessive and repeated application of P fertilizer to cropland (Sadia Alam *et al.*, 2002).

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Therefore, the present study was carried out the isolation and phosphate solubilizing activity of isolated bacteria from agricultural field. The aim and objectives of this paper are to isolate the phosphate solubilizing bacteria (PSB) from soil, to study phosphate solubilizing activity of isolated bacteria and to investigate the effect on *Vigna mungo* L. (Blackgram) of six selected phosphate solubilizing bacteria.

Materials and Methods

Collection of Soil Samples

Four different soil samples were collected from four different places of Taung-Yar-Kone village in Pathein Township, Ayeyarwady Region in Myanmar.

This experiment was carried out at the laboratory of Biotechnology and Development Center of Pathein University.

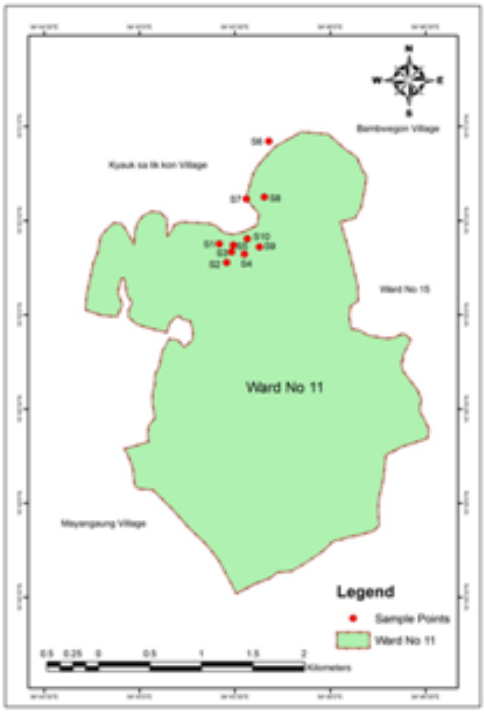


Figure (1) Map of Soil Samples Collected Area in Pathein Township, (Source-Geography Department, Pathein University)

Preparation of Glass wares

Pyrex glass wares were used throughout the experiments. The glass wares were treated with the chromosulphuric acid and washed them with water. After air drying, they were sterilized in an autoclave at 15 psi and 121°C at 15 minutes.

Table (1) Four different soil samples collected from Pathein Township.

Soil sample	Collected places	Soil type	Soil pH	Moisture %	location
S-1	Taung-Yar Kone Village	Sandy Loam	6.09	2.46	16°49.833"N 94°45.572"E
S-2	Taung-Yar Kone Village	Sandy Loam	6.18	1.37	16°50.341"N 94°45.462"E
S-3	Taung-Yar Kone Village	Loam	6.87	1.53	16°50.349"N 94°45.498"E
S-4	Taung-Yar Kone Village	Loam	6.89	2.15	16°50.866"N 94°45.522"E

Serial Dilutions Methods of Soil Samples

Serial dilutions of fermented, plating and streaking techniques described by Salle (1948) Collins (1965) and Pelezer and Chan (1972) were used for the isolation of microorganisms from soil. An appropriate amount (1 gm) 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^{-1} to 10^{-5} dilution in separate test tubes and 1ml each of the above dilutions was separately transferred into sterile petridishes under aseptic condition. A sterile pipette was used for each transfer. The sterilized medium in conical flask was cooled down to about 45°C and separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anti clock-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. They were separately streaked over another set of petridishes containing the same sterile medium. Each of the discrete colonies visible in the second set of inoculated plates was separately transferred to sterile nutrient agar medium. The isolates were maintained in nutrient agar medium for further experimentations.

Isolation procedure for soil sample

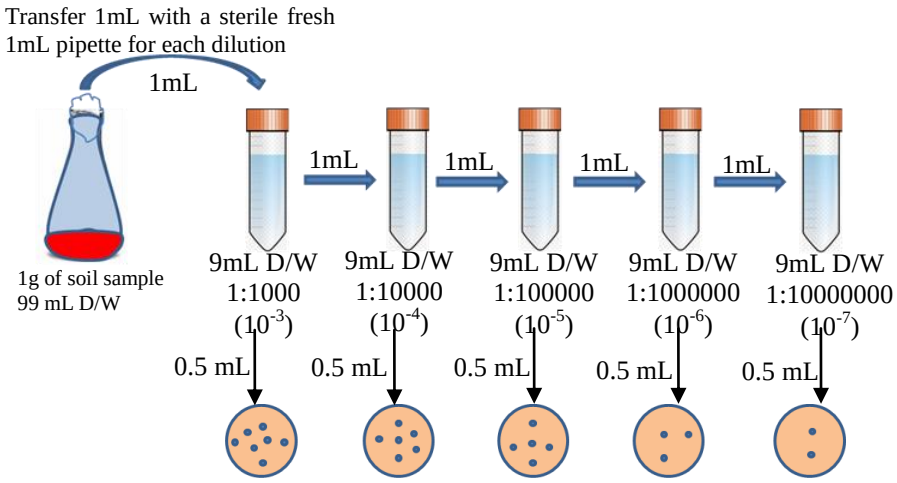


Figure (2) Serial Dilution Method (Collin, 1965).

Media used for isolation of Bacteria

Pikovskaya medium (PVK) (Subba Rao, 1977)

Glucose	10g
$Ca_3(PO_4)_2$	5 g
$(NH_4)_2\ SO_4$	0.5 g
NaCl	0.2 g
$MgSO_47H_2O$	0.1 g
KCl	0.2 g
Yeast extract	0.5 g
$MnSO_47H_2O$	0.002 g
$FeSO_47H_2O$	0.002 g
$C_{27}H_{28}Br_2O_5S$	0.008 g
Agar	15 g
Distilled water	1000 mL
pH	6.8-7

After autoclaving, Nystatin (1.5 mL) was added to the medium.

Preliminary Study of Phosphate Solubilizing Bacteria

Preliminary test for phosphate solubilizing bacteria of the starins isolated from the soil samples of different places (Taung-Yar-Kone village) were studied. One day old culture media was used to inoculate with each of all isolated bacteria from soil samples. Two days after inoculation, the clear halo zone appeared around the spot culture indicated the inoculated bacteria showing the capability of phosphate solubilization. The organisms showing the phosphate solubilize activities were selected for the further studies of morphological characteristic and various chemical tests for identification.

Morphological Characteristics and Staining Reactions of Isolated Bacteria

Gram Staining

A drop of sterile distilled water was placed on clean grease-free slide and a small loop of isolated bacteria was smeared on the slide and allowed it to dry. The smear was fixed by passing the dried slide 3 or 4 times rapidly over a flame. The slide was covered with crystal violet stain and allowed it to act for 30-60 seconds. Then, the slide was rinsed with distilled water for a few seconds. The slide was covered with fresh iodine solution and allowed it to act for about 30-60 seconds. The alcohol drop was added until no more colour flowed out from the smear. For a thin smear 10-20 seconds may be enough for complete decolourization of gram negative bacteria. As a counter stain, the smear was covered with safranin for about 20-30 seconds and washed with distilled water. Then the slide was air dry. The strained slide was examined under the oil immersion objective of the microscope (Atlas 1993).

Phosphate Solubilizing Activity

From the isolate, halozone producing strains were selected for further study. The phosphate solubilizing activity of the selected isolates was conducted by plate screening method on Pikovskaya's medium. Isolate showing phosphate solubilizing ability was spot

inoculated at the centre of Pikovskaya's plate and incubated at 37°C. Diameter of clearance zone was measured sucessively after 24 hours upto 7 days culture. The Phosphate Solubilization Efficiency (PSE) is the ratio of the total diameter.

$$PSI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

*PSI = Phosphate Solubilizing Index

Preparation of Seed Germination

Plastic cell-trays were used as seed germination test. One seed was placed in each cell (1.5 cm in diameter) One strain was laid out with 4 replications. Each replication contained 25 seeds. Fermentation broth was sprayed once a week, then water was sprayed daily to maintain the soil moisture of seed beds. The culture of 3 days old in broth was diluted into distilled water 2:8 and applied onto the plants as biofertilizer suspension. This study was conducted in Pathein University Campus, during November, 2017.

Determination of Seed Germination and Survival Percentages

The percentages of seed germination and survival were calculated by the following formula (ISTA, 1976).

$$\text{Germination (\%)} = \frac{\text{Total number of germinated seeds}}{\text{Total number of the sown seeds}} \times 100$$

$$\text{Survival (\%)} = \frac{\text{Total number of survival plants}}{\text{Total number of the sown seeds}} \times 100$$

Data Collection

Shoot length, seed germination and survival percentages were recorded. The germination percentages were determined at one weeks (7 days) after sowing seeds (7 days). The survival percentages were determined within two weeks (14 days) after sowing seeds (14 days).

Results

Colony Morphology of Isolated Phosphate Solubilizing Bacteria

Soil samples were collected from four different soils and 19 bacterial strains were obtained. In the colony morphology, isolated bacteria were small, medium and large in size of colony and the colours were white, cream, yellow, pale yellow, yellowish orange and transparent white. The margins of the bacterial colony were entire. The elevation and form were raised and flat. The isolated bacterial strains were rod, short rod and cocci. Among them, 14 strains were gram negative and 5 strains were gram positive.

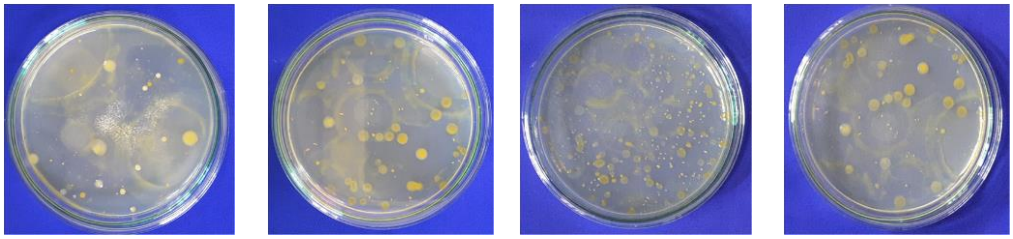
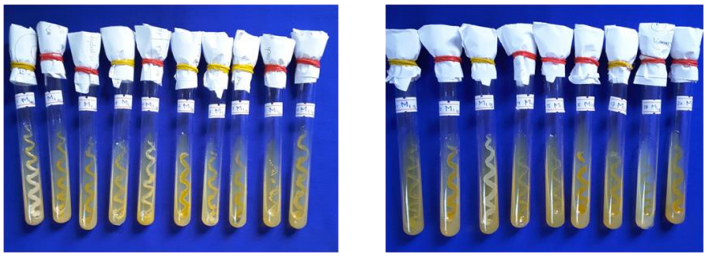


Figure (3) Colony morphology of bacteria from four soil samples S-1, S-2, S-3 and S-4.



MMT 1-10 MMT 11-19

Figure (4) Preservation of Isolated Bacteria on Slants

Table (2) Colony Morphology of Isolated Bacteria.

Bacteria No.	Size of Colony	Margin	Color	Elevation and Form	Pigment on agar	Cell Morphology	Gram Staining
MMT-1	Mediu	Entired	White	Raised	White glistening	short-rod	(+)
MMT-2	Mediu	Entired	Pale Yellow	Raised	Yellow glistening	short-rod	(-)
MMT-3	Small	Entired	Cream	Raised	Cream glistening	short-rod	(+)
MMT-4	Small	Entired	Yellowish orange	Raised	Yellow glistening	rod	(-)
MMT-5	Mediu	Entired	Pale Yellow	Flat	Yellow glistening	cocci	(-)
MMT-6	Small	Entired	Yellowish orange	Raised	Yellow glistening	rod	(-)
MMT-7	Small	Entired	Yellow	Raised	Yellow Pigment	cocci	(-)
MMT-8	Small	Entired	Brown	Raised	Brown Pigment	rod	(-)
MMT-9	Small	Entired	Pale Yellow	Raised	Yellow glistening	short-rod	(-)
MMT-10	Small	Entired	Cream	Raised	Cream glistening	short-rod	(-)
MMT-11	Small	Entired	Cream	Raised	Cream glistening	short-rod	(-)
MMT-12	Mediu	Entired	Pale yellow	Raised	Yellow glistening	rod	(-)
MMT-13	Small	Entired	White	Raised	White glistening	short-rod	(+)
MMT-14	Mediu	Entired	Transparent	Raised	White glistening	short-rod	(+)
MMT-15	Mediu	Entired	Pale yellow	Raised	Yellow glistening	rod	(-)
MMT-16	Small	Entired	Yellowish orange	Raised	Yellow glistening	cocci	(+)
MMT-17	Large	Entired	Pale yellow	Raised	Yellow glistening	cocci	(-)
MMT-18	Small	Entired	Yellowish orange	Raised	Yellow glistening	rod	(-)
MMT-19	Small	Entired	Yellow	Flat	Yellow pigment	short-rod	(-)

Small < 2 mm(diameter)

Medium = between 2 mm and 5 mm (diameter)

Large > 5 mm (diameter)

(+) = Gram-positive

(-) = Gram-negative

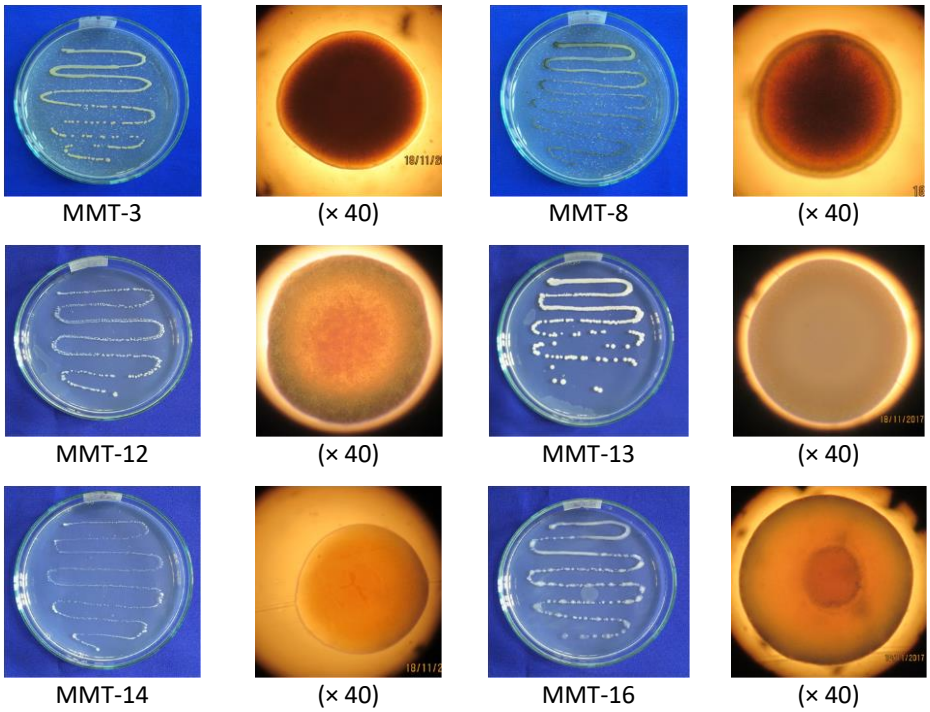


Figure (5) Colony and Microscopical Character of Selected Bacteria, MMT-3, 8, 12, 13, 14 and MMT-16.

Phosphate Solubilizing Activity of Selected Bacteria

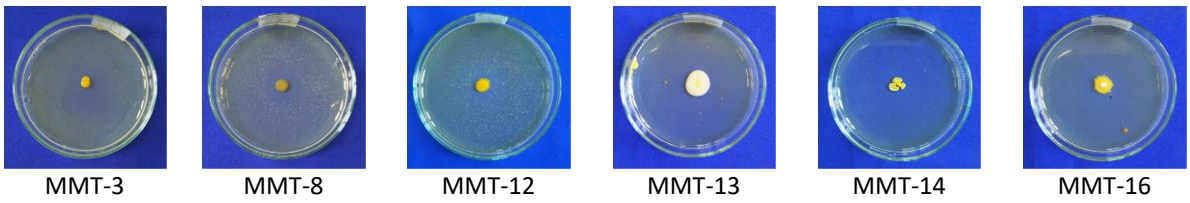


Figure (6) Halozone Around the Colony on PVK Media Confirm, Phosphate Solubilizing Bacteria

Phosphate solubilizing activity of six selected bacteria

Out of 19 bacteria, six strains showed the halozone. Among these potent isolates, MMT-8 showed (4.14 mm) followed by MMT-12 (2.50 mm).

Table (3) Measuring the Diameter of Clearance Zone, Phosphate Solubilizing Bacteria.

Selected	Colony diameter	Halo zone diameter	PSI (mm)
MMT-3	8	20	3.50 mm
MMT-8	7	22	**4.14 mm**
MMT-12	8	12	2.50 mm
MMT-13	15	23	2.53 mm
MMT-14	8	15	2.87 mm
MMT-16	13	26	3.00 mm

*PSI – Phosphate Solubilization Index

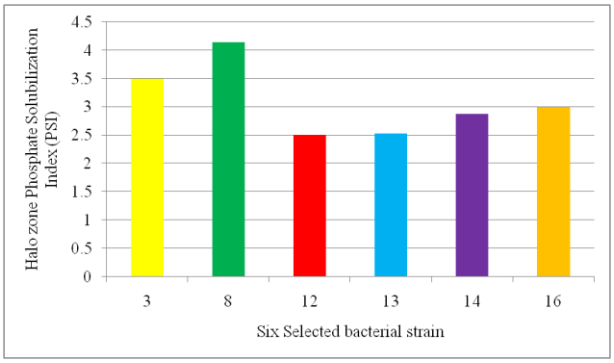


Figure (7) Different Clearance Zone (halo zone) of six bacterial strains on PVK Medium.

Seed germination of Black gram (7 days)

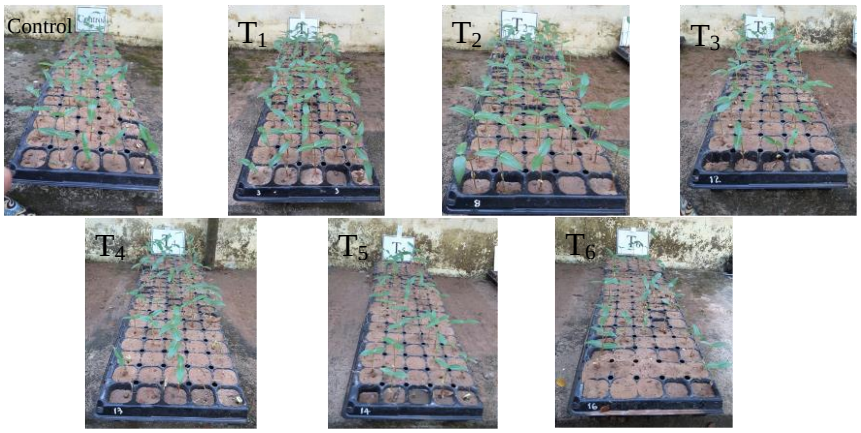


Figure (8) Effect on seed germination of blackgram with the treatment of six selected bacteria

Survival of Black gram (14 days)

Figure (9) Effect on the survival of blackgram with the treatment of six selected bacteria.

Plant Height

There was a highly significant difference in the means of plant height among the selected phosphate solubilizing bacteria as 7 days and 14 days. Among the selected bacterium, the tallest plants were observed in T_1 (MMT-3) after 7 days (9.54 cm) and 14 days (19.52 cm) (Table 4 and Figure 10). The shortest plants were observed in control after 7 days (5.63 cm) and 14 days (12.02 cm).

Table (4) Maximum Plant Height of Selected Phosphate Solubilizing Bacteria

No.	Selected bacterial strains	Plant Height (Shoot length)	
		7 days (cm)	14 days (cm)
1	Control	5.63	12.02
2	MMT-3 (T_1)	9.54	19.52
3	MMT-8 (T_2)	8.70	18.70
4	MMT-12 (T_3)	8.10	17.78
5	MMT-13 (T_4)	6.68	15.52
6	MMT-14 (T_5)	7.41	16.6
7	MMT-16 (T_6)	6.61	15.0

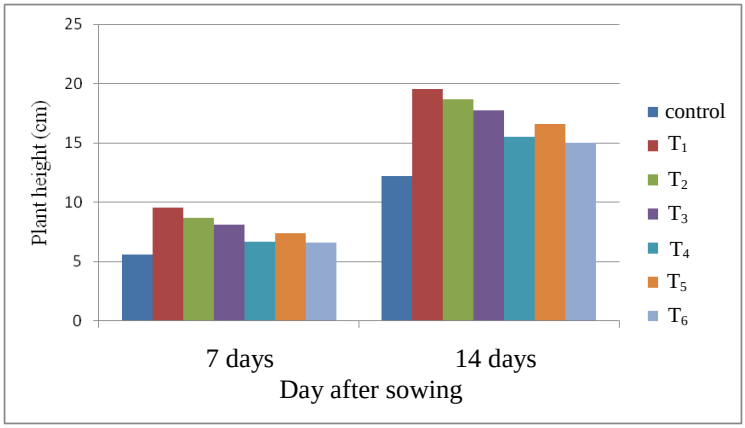


Figure (10) Plant Height of the Selected Bacterium at 7 days and 14 days

Table (5) Seed Germination and Survival Percentages after 7 days and 14 days.

No.	Selected bacterial strains	Seed germination (%)	Survival (%)
		7 days	14 days
1	Control	80	84
2	MMT-3 (T_1)	92	92
3	MMT-8 (T_2)	88	88
4	MMT-12 (T_3)	76	80
5	MMT-13 (T_4)	76	76
6	MMT-14 (T_5)	80	80
7	MMT-16 (T_6)	84	84

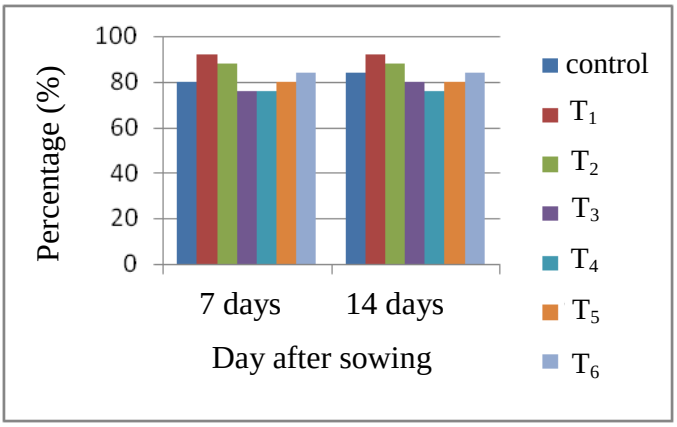


Figure (11) Seed Germination and Survival Percentage of the Selected Bacterium after 7 days and 14 days.

Discussion and Conclusion

Phosphorus is one of the major limiting factors of crop production on many tropical and subtropical soils. It is therefore necessary to identify and incorporate efficient strains of phosphate solubilizing microorganisms into cropping systems (Fankem *et.al.*, 2006). For sustained agricultural production, the use of efficient fertilizer to maintain the soil and plant quality is critical. The application of organic fertilizer has been practiced for more than thousand years in many countries since it provides essential nutrients to plants, improves soil structure, helps in the moisture retaining capacity in various soils and increases microbial activities (Chen *et.al.*, 2006).

Four different soil samples were collected from four different places at Pathein Township during July and August, 2016. After that day, these soil samples were isolated by serial dilution method on Pikovskaya medium (PVK). A total of 19 bacterial colonies were obtained. The isolated bacterial strains were designated as MMT 1-19. MMT 1-7 were isolated from soil sample 1 (Sandy loam), MMT 8-13 from soil sample 2 (Sandy loam), MMT 14-15 from soil sample 3 (loam), MMT 16-19 from soil sample 4 (loam).

In the colony morphology, the isolated bacteria were small, medium and large in size and the colours were white, pale yellow, yellow, cream and brown. The margins of colonies were entire and the elevations were raised and flat. Six strains were rod, nine strains were short rod and four strains were cocci.

Sharma (2011) reported that the morphological features of PSB colonies were with irregular margins, rod shaped, gram positive and gram negative from agricultural soil.

The halozone of six bacterial isolates were studied and among them, the highest Phosphate Solubilization Index was found in MMT-8 (4.14 mm) and MMT-3 (3.50 mm).

These findings were also supported by Ahamad & Jha (1967) that the phosphobacteria were identified by noting the solubilizing zone formed around the bacterial colony.

In the study of seed germination and survival percentages of *Vigna mungo* L. (Blackgram), six selected phosphate solubilizing bacteria were used as the treatment on Blackgram.

In this result, seed germination and survival were the maximum percentage (92%) on treatment 1 (MMT-3) followed (88%) in treatment 2 (MMT-8) both after 7 days and 14 days.

Phosphorus solubilizing bacteria (PSB) play an important role by enhancing its availability to plants through release from inorganic and organic soil Phosphate by solubilization and mineralization (Kumar and Bharathi, 2015).

In the study on plant height of Blackgram, the tallest plants were observed in T1 (MMT-3) after 7 days (9.54 cm) and 14 days (19.52 cm). The shortest plants were observed in control after 7 days (5.63 cm) and 14 days (12.02 cm).

Babannavar (1990) described that growth and yield of crop plants are influenced by the presence of sufficient quantities of nutrients in available form for plant uptake.

Acknowledgment

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References

Abdalla, M.H. (1994). Phosphatases and the utilization of orgnaic phosphorus by Rhizobium legum in osarum biovarviceae. Lett. Appl. Microbial, 18, 294-296.

Ahmad Ali Khan, GhulamJilani, *et.al.,* (2009). Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. Journal of Agricultural Biological science, 11:48-58.

Ahmad, N. & K. K Jha. (1967). Solubilization of rock phosphate by microorganisms isoalted from Bihar soils.Journa; pfGemera; Amd Applied Microbiology, 14:89-95

Amit Sagervanshi, *et.al.,* (2012). Isolation and Characterization of Phosphate Solublizing Bacteria from Anand Agriculture Soil, International Journal of Life Sciences and Pharma Research, 23;256-266.

Atlas, R.M. (1993). Microbiology Fundamentals and Application. Mecmillan Publishing Co., a division of Macmillan, Inc.

Babannavar PB (1990). Response of sunflower (*Helianthus annuus* L.) to varying levels of nitrogen and phosphorus under saline water irrigation. MSc thesis, University of Agricultural Sciences, Dharwad, 135 pp.

Chen *et al.,* (2006). Phosphate Solubilizing Bacteria from surface soil and their tricalcium phosphate solubilizing abilities. Appl. Soil. Ecol., 34:33-41

Collins, C.H. (1965). Microbial methods. Butterworth & Co., Publishers Ltd., London.

Fankem *et.al.,* (2006). Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree rhizosphere in Cameroon. African journal of Biotechnology, 5: 2450-2460

ISTA. International Seed Testing Association. (1976). Handbook of Vigor Test Method.

Kannapiran, *et.al.,* (2011). Isolation of phosphate Solubilizing bacteria from sediments of Thondi coast, Palk Strait, Southeast coast of India.*Annals of Biogical Research,* 25:157-163.

Kumar and Bharathi, (2015). Screening the effect of phosphate solubilizing Bacteria on Black Gram (*Vigna mungo* L.) International Journal of Science and Research (IJSR) ISSN (online): 2319-7064.

Pelazar, M. J. & E. C. S. Chan. (1972). Exercises in microbiology.3rd ed. McGraw. Hill Book Co., New York.

Sadia Alam, *et.al.,* (2002). In vitro solubilisation of inorganic phosphate by phosphate solubilizing microorganisms PSM from maize rhizosphere.*International Journal of Agriculture and Biology*. 4:454-458.

Salle, A. J. (1948). Fundamental principles of bacteriology. Mc. Graw Hill Book Co., Inc. New York.

Subba Rao N.S., (1977). Soil Microorganisms and Plant Growth, Oxford and IBH Publishing Co., New Delhi.

Sharma S. *et al., (*2011). Isolation of Phosphate Solubilizing Microorganism (PSMs) From soil. J. Microbiol. Biotech. Res., 1 (2): 90-95.

Appendix

DEPARTMENT OF AGRICULTURE (LAND USE)

SOIL ANALYTICAL DATA SHEET

Division - ရောဝတီ ခေါ်မြင့်မြင့်သန်း (၅.၇.၂၀၁၆) Sheet No. 1

Township - ပုသိမ် Sr No. S 1-5/16-17

Sr No.	Sample plot	Moisture %	pH Soil: Water 1: 2.5	Texture				SOIL INTERPREATATION OF RESULTS	
				Sand %	Silt %	Clay %	Total %	pH Soil: Water 1: 2.5	Texture
1	MT-01	2.46	6.09	55.25	24.50	19.20	98.95	Slightly acid	Sandy loam
2	MT-02	1.37	6.18	54.55	31.70	12.20	98.45	Slightly acid	Sandy loam
3	MT-03	1.53	6.87	46.65	35.15	16.55	98.35	Near Neutral	Loam
4	MT-04	2.15	6.89	49.00	28.30	21.60	98.90	Near Neutral	Loam