Eco. Env. & Cons. 26 (February Suppl. Issue) : 2020; pp. (S53-S61)
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ISSN 0971–765X

Isolation and characterization of phosphate solubilizing bacteria from rhizosphere soils for potential use in agricultural practices of *Solanum melongena*

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(Received 31 October, 2019; accepted 26 November, 2019)

ABSTRACT

The ability of some soil microorganisms to convert insoluble forms of phosphorus to an accessible form is crucial in plant growth-promoting bacteria for increasing plant yield. Phosphate Solubilising Bacteria (PSB) as inoculants increases the P uptake by plants. They interact with root region and enhance the plant growth directly or indirectly. In this study, twelve rhizosphere soil samples of *Solanum melongena* were collected from three different places and characterized the potential PSB which solubilised organic P in Pikovaskaya media, rock phosphate and eggshells and bones was identified. The potential strain was isolated and characterized as *Rhizobium* sp. This strain was inoculated in rhizosphere to increase the plant growth as well as crop yield. The effect of the PSB on growth of *S. melongena* seedlings were studied in pot experiment. The plants parameters results showed the highest mean shoot length of *S. melongena* was achieved by the condition of *Rhizobium* sp. with eggshells and bones application at 14.00 cm, followed by *Rhizobium* sp. with Triple Super Phosphate (13.00cm) and finally *Rhizobium* sp. with Rock Phosphate (11.60cm). Moreover, the chlorophyll content of *S. Melongena* showed maximum value for the condition of *Rhizobium* sp. inoculated with eggshells and bones application (6.215mg/g), followed by PSB inoculated with TSP (5.932 mg/g) and finally inoculated with RP (5.214mg/g). So, it can be concluded that rock phosphate simultaneously combined with PSB strain tends to solubilise organic P in the soil as good as TSP and they also can be used as alternative chemical phosphate fertilizer. Besides that, eggshells and bones were used as substitutes of inorganic P to study the ability of PSB to solubilise the inorganic P.

Key words : *Phosphate solubilizing bacteria (PSB), Solanum melongena, Triple super phosphate, Rock phosphate, Eggshells and bones.*

Introduction

Phosphorus (P) is the major macronutrients that crucial for energy storage and transfer reactions in plants. They are applied into the soil in the form of phosphatic fertilizers and most of the P compounds are found in soluble or insoluble formto enhance the plant growth (Sims and Sharpley, 2005). Usually, the inorganic phosphate that applied to the soil immobilized rapidly. This phenomenon causes the plant unavailable of the nutrients from the soil (Goldstein, 1986). Thus, researchers have considered a range of bacterial species such phosphate solubilising bacteria from rhizosphere of the plant to solubilise the insoluble form of P into soluble P to increase the P availability to the plants as a major nutrient (Rodriguez and Fraga, 1999). These types of microorganisms are known as 'plant growth promoting rhizobacteria' (PGPR). PGPR perform their role either directly or indirectly to enhance the plant growth (Glick, 1995). Direct growth is the PGPR increase the mineral nutrient solubilisation and nitrogen fixation by alter the plant hormones through phytohormones synthesis, N fixation, and limits the membrane potential of the root (Xie *et al.,* 1996; Christiansen, 1992). Besides that, enzymes such 1-aminocyclopropane-1-carboxylate (ACC) deaminases are produced against the plant stress tolerance. Alternatively, the inorganic phosphates are solubilised to organic form and it easily obtained by the plants. However, the indirect growth is the deleterious or repression of pathogenic microorganisms by production antibiotics, hydrogen cyanide or siderophores (Sivan and Chet, 1992; Leong, 1986).

It is well known that soil is mixed compound of several types of commercial phosphate fertilizers to develop the soil fertility (Bhatti and Yawar, 2010). The approximate level of Phosphorus (P) in soil is 400-1200 mg.kg{ . The structure of P compound allows it to react with other ions such hydrogen (Hz), potassium (Kz) and ammonium (NHz). In addition, P is absorbed in the soil in form of H_2PO_4{ or HPO_4^{2-} with very low concentration. Anyhow, the exchange of the ions in the soil is depends on the soil pH. The optimum pH of the soil is 7 where the calcium phosphate minerals can solubilise to high level and providesufficient P to the plants. At the range of 5.0 – 8.0 pH, the phosphates react efficiently with other cations to form stable compounds that can produce P. Any pH value below 7 indicates the soil is acidic and above 7.5 is alkaline. In acid soils, P fertilizers solubilised in low rate due to the calcium fixationin the soil (Goldstein, 1994; Goldstein and Liu, 1987; Jones *et al.,* 1991). Furthermore, researchers have proved that rock phosphate and other appetites are major source of phosphorus in geological age(Fernandez and Novo, 1988) recently, rock phosphates are achieving high demand in soil management. For example, in Malaysia, Christmas Island Rock Phosphate one of the ground rock phosphates has been used for plantation such as rubber and oil palm (Zaharah *et al.,* 1985). Egg shell contains 1 5 of calcium phosphate (1%) and 94 % calcium carbonate (Rivera *et al.*, 1999). Even egg shell can be used as a source of P as plants requires less amount of 'P'.

The main aim of this research is to study the rhizosphere effect of PSB on *Solanum melongena*and to isolateand characterize the potential PSB from rhizosphere soil. By considering the problem of P fertilizer cost and leaching effect due to the high rain fall in Malaysia, we intend to use insoluble eggshells and bones as 'P' fertilizer. Though P source in the selected PSB strain inoculated to the *S.melongena* and their effect were studied.Besides that, we intend to investigate the solubilisation efficiency of the selected PSB strain in the context of eggshells and bones. In order to study the potential effect of PSB on the selected plants, various plant and yield parameters need to study. In addition, the organic productions of vegetables are more attractive in these days. So, in this study we step into the partial organic production of vegetables by eggshells and bones.

Materials and Methods

Materials

Eggplant (*Solanum melongena*) seed was obtained from local market. Pikovskayaagar/ broth medium was obtained from HiMedia. All the chemicals obtained were from HiMedia and Rankem. All the preparations were done in distilled water. Tap water was used for plants.

Preparation of rhizosphere and non-rhizosphere sample

The root of the eggplant has removed out from the soil and gently shaken to remove the loosely attached soil. Both soils (removed and still attach to the root) were sealed in zip-lock plastic bag. They were labelled accordingly as rhizosphere (adhere to root) and non-rhizosphere (removed soil) which were than stored at 4 ^{o}C until use.

Isolation of Phosphate Solubilising Bacteria

1g of rhizosphere and non-rhizosphere soil samples were weighed and mixed in 100 mL of sterile saline water in different conical flasks. These were kept on shaker for 30 min for vigorous mixing at room temperature and serially diluted up to 10^{-8} fold with 9mL of saline water in each test tube. 100 µL of diluted samples were spread on PK media and incubated at 37 ^{0}C for 3 days.The solubilisation efficiency of the PSB colony calculated as follow

$$\text{Solubilisation efficiency} = \frac{\text{Solubilisation Diameter (Z)}}{\text{Growth Diameter (G)}} \times 100$$

Evaluation for phosphate solubilisation

The positive phosphate solubilising bacterial colonies that isolated in the PK media were evaluated. The screening method was carried out to compare the solubilisation efficiency with the other colonies. Finally, the potential colony with high solubilisation efficiency was selected for further characterization.

Characterization of selected colony

The high efficiency P solubilizing bacteria were performed with various biochemical tests such gram stain, motility test, catalase test, oxidase test (Sood, 2006) and endospore stain with starch hydrolysis were carried out (Willey *et al.*, 2008).

Inoculum preparation and root inoculation

Selected strain of PSB was inoculated in 100ml of nutrient broth under aseptic condition and incubated at 37^{o}C for 24 hours and centrifuged at 5000rpm for 15 min. Pellets were resuspended in 150ml of sterile tap water. The germinated seedlings were picked out and the root region washed with tap water and then inoculated with the inoculums by dip into the inoculums that prepared (Anand, 2012).

Experimental plan

Experiments were conducted with total of 24 pots; 8 rows and each row consist 3 pots. Each of the pot filled up with approximately 4kg of soils. Furthermore, each pot has planted with 2 eggplant seedlingsand has labelled as A to H. A) Control uninoculated without any fertilizer – Environmental control, B) Control uninoculated + TSP, C) Control uninoculated + RP, D) Control uninoculated + bones and eggshells, E) PSB strain + without fertilizer, F) PSB strain + TSP, G) PSB strain + RP, H) PSB strain + bones and eggshells. (TSP – Triple Super Phosphate, RP – Rock Phosphate, PSB – Phosphate Solubilising Bacteria).

Fertilizer application

80g urea + 80g potash dissolved in 400mL of tap water and distributed to each pot as 20 mL (B,C,D,F,G,H), 20g of TSP dissolved in 200mL of tap water and distributed into each pot 20 mL (B& F). 20g RP dissolved in 200 mL tap water and distributed into each pot 20 mL (C&G). Eggshell + Bones (powder form) distributed into each pot as 30g (D & H).

Plant parameter Analysis

The plant parameters (shoot length, number of the leaves, number of flowers, number of fruits together with the diameter and weight) were measured. The parameters were taken every 15 days (Cornelissen *et al.,* 2003). Total chlorophyll content estimation was carried out according to Trivedy and Goel (1986).

Bone experiment

Experiment was designed as follows : P – Condition - Eggshells only (E), Bones only (B) andP ^{10} Condition - Eggshells + P^{10} (EP), Bones + P^{10} (BP). P^{10} stands for 0.05g/100 mL of $Ca_3(PO_4)_2$(10% of total phosphorus in modified PK media)

In order to study the phosphorus solubilisation of selected strain, the modified PK broth was used. Two sets of PK broth were prepared where the first set contains 25% (0.05g/100mL) of inorganic phosphorus and another set consist 0% (zero phosphorus) of inorganic phosphorus as control. After sterilisation, the selected strain inoculated and incubated at 37 ^{0}C at 100rpm rotation. 1 mL of samples were taken every six hours interval and soluble P were estimated using stanneous chloride method (Carson, 1976; Kuttner and Lichtenstein, 1930).

Results

Physico –Chemical parameters of soil

Soil was subjected for chemical parameters and the obtained results are illustrated in Table 1.

Rhizosphere effect of PSB in *Solanum melongena* plant from different location.

The collected samples were subjected for isolating PSB on PK media. The colony forming unit (CFU/g) of PSB is greater at rhizosphere soil compared to non-rhizosphere soil (Table 2). It indicates the rhizosphere effect is higher at rhizosphere soil than the non-rhizosphere.

Table 1. The physico-chemical parameters of the soil from FRIM (Forest Research Investigation of Malaysia) for cultivation

Chemical components	Units
pH	7.65
Organic carbon	3.26%
Organic nitrogen	0.10%
Available P	2115 ppm
Exchangeable Ca	4.66 c mol/kg
Exchangeable Mg	2.72 cmol/kg
Exchangeable	2.62 cmol/kg

Total number of PSB isolated from the rhizosphere soil samples.

The PSB strains were isolated and the colonies were labelled with specific range of reference numbers for further identification (Table 3).

Enrichment results

Colonies that form halo zone (clear zone) in PK media were considered as high efficiency bacteria that solubilise P / Phosphate solubilising bacteria. Among the isolated PSB, the colony number PSB49 has showed high solubilisation efficiency (Table 4). Thus, this strain has selected as potential strain and the various biochemical tests were performed for further identification.

Biochemical Tests

From the above solubilisation efficiency that performed for the various strain, PSB49 were selected due to the high solubilisation efficiency. Thus, the strain was identified with biochemical tests and confirmed as *Rhizobium* sp.

Plant Parameters Analysis

The pot study results showed that the inoculated

Table 2. Total PSB isolated from both rhizosphere and non-rhizosphere samples.

Area	Serial numbe of soil samples	Phosphate Solubilizing Bacteria (PSB) (CFU/g)	
		Rhizosphere	Non -Rhizosphere
Labu	L-1	4.18 x 10^7	5.03 x 10^5
	L-2	4.76 x 10^6	4.89 x 10^5
	L-3	4.65 x 10^6	3.07 x 10^6
	L-4	5.19 x 10^7	4.43 x 10^7
	L-5	3.45 x 10^7	3.38 x 10^6
	L-6	5.12 x 10^8	3.21 x 10^8
SertingHilir	S-1	5.20 x 10^6	6.61 x 10^5
	S-2	6.23 x 10^6	5.87 x 10^8
	S-3	5.42 x 10^6	3.62 x 10^7
KotaDamansara	K-1	5.80 x 10^6	3.33 x 10^9
	K-2	6.30 x 10^7	4.27 x 10^6
	K-3	5.14 x 10^8	4.09 x 10^6

Table 3. Total number of PSB isolated on Pikovskaya (PK) media

Area	Total Number of samples	Number of PSB strains isolated	Reference Strain Number of bacteria
Labu	1	3	PSB 1, 2, 3
	2	5	PSB 4, 5, 6, 7, 8
	3	4	PSB 9, 10, 11, 12
	4	5	PSB 13, 14, 15, 16, 17
	5	3	PSB 18, 19, 20
	6	2	PSB 21, 22,
SertingHilir	1	5	PSB 23, 24, 25, 26, 27
	2	6	PSB 28, 29, 30, 31, 32, 33
	3	5	PSB 34, 35, 36, 37, 38
Kota Damansara	1	4	PSB 39, 40, 41, 42
	2	2	PSB 43, 44
	3	8	PSB 45, 46, 47, 48, 49, 50, 51, 52, 53

bacteria *Rhizobium sp.* has act on *Solanum melongena* seedlings. The results showed the expected positive rhizosphere effect on bacterial inoculated plants compared to the uninoculated plants (Table 5). Plant parameters such as shoot length and root length of both inoculated and uninoculated shows the obvious differences.

To study the effect of PSB strain (*Rhizobium sp.)* on *S.melongena,* the plant parameters were taken on 40^{th} and 55^{th} days of seedlings. For the first parameter, plant mean shoots length (cm), mean number of leaves, and mean number of flowers was taken (Table 5). However, after 55 days of seedlings, the plant starts to fruiting, few more parameters such mean number of fruits, mean length of fruits (cm), and mean diameter of the fruits (cm) also were performed (Table 5). In addition, mean weight of the fruits has been measured (Table 5).

Mean shoot length (cm) of inoculated and uninoculated plant conditions were compared (Table 5). There are varies differences in the shoot length (cm) of inoculated and uninoculated. The inoculated plants show higher value especially pot inoculated with eggshells and bones than the uninoculated. Whereas, the lowest value achieved by inoculated +TSP application. This is due to the low solubility of the fertilizer into the soil. Moreover, the mean shoot length of uninoculated pots is maintained constantly whereas the inoculated pot shows different values at different conditions.

Mean number of leaves of PSB inoculated pot and uninoculated pot was compared (Table 5). Both are showing constantly increasing. However, the PSB inoculated pot achieved higher mean value compared to untreated. Eventually, both the uninoculated and inoculated pots showing high means value for the eggshells and bones application

Mean number of flowers of *Rhizobium* sp. inoculated and uninoculated pots were studied (Table 5). The control environmental of uninoculated pot showed lowest mean value and the highest value at eggshells and bones. Yet, the PSB inoculated pots showing highest mean value at eggshells and bones application and lowest for rock phosphate application.

Measurement of plant parameters after 55 days

Mean shoot length (cm) were measured again after 55 days of seedlings (Table 6). The results are showing the same higher value for inoculated pots than the uninoculated pots. For the PSB uninoculated pots, the values are remaining constant whereas for PSB inoculated pots the values are increasing.

Mean number of leaves showing higher value

Table 4. Solubilisation efficiency of the strains in PK media

Strain Num	Colony diameter (mm)	Halo Zone (mm)	Solubilisation Efficiency (%)
PSB2	4.0	4.5	4.5 / 4.0 X 100= 112.5
PSB6	5.0	3.5	3.5 / 5.0 X 100= 70.0
PSB19	5.5	4.5	4.5 / 5.5 X 100= 81.8
PSB25	4.5	4.0	4.0 / 4.5 X 100= 88.90
PSB36	5.0	4.5	4.5/ 5.0 X 100= 90.0
PSB49	3.5	3.0	3.0 / 3.5 X 100= **116.67**

Table 5. Results of *S. melongena*parameters after 40 days of seedlings

Pot Experimental Conditions	Mean Shoot Length(cm)	Mean Number of Leaves	Mean Number of Flower
A	10.89	10.11.	1.30
B	11.20	9.80	1.80
C	10.66	11.33	2.00
D	11.00	12.10	2.20
E	13.23	11.20	1.90
F	13.00	11.10	2.20
G	11.60	13.00	1.90
H	14.00	14.10	2.90

A: Uninoculated without fertilizer, E-Inoculated without fertilizer, B - Uninoculated + TSP, F - Inoculated + TSP, C - Uninoculated + RP, G - Inoculated + RP, D - Uninoculated + Bones and Eggshells, H - Inoculated + Bones and Eggshells than the 40 days of result (Table 6). In contrast, the TSP applications for both inoculated and uninoculated pots showing nearly similar value. However, both conditions show highest mean value of leaves for eggshells and bones application. The fruit parameters were performed to compare the PSB effect on inoculated pot and uninoculated pot. The mean number of fruits,mean length of fruits (cm) and mean diameter of fruits were performed against the rhizosphere effect. The three parameters are clearly showing high mean value at PSB inoculated pot than the uninoculated pot (Table 6).

The mean weight of the fruits for each plant conditions (A to H) was measured (Table 6) to study the PSB effect on yield production. Figure 21 shows the graph as comparison of yield production for each plant conditions.

Based on the results above (Table 6), the yield production (mean fruit weight) of both inoculated and uninoculated plants showing constantly increasing. However, plants with PSB inoculated showed greater mean value compared to uninoculated. This indicates the rhizosphere effect is positive towards *S. melongena* in enhancing the crop production. Subsequently, based on statistical analysis, 95% confidence that the P value is <0.05. Thus, the statistical analysis is significant.

Chlorophyll Measurement

Chlorophyll measurement is to measure the leaf pigments which play a valuable role in plants. The chlorophyll content was studied at different wavelength; 663nm and 645nm using UV spectrophotometer. The results of chlorophyll a and b weresummed up for every pot of leaf (Table 6).

Based on the table above, the chlorophyll content in inoculated plants are higher than the uninoculated plants. This indicates the positive effects towards the PSB inoculated plants which means that bacteria had carried out more photosynthetic reaction compared to the uninoculated plants.

Bone experiment

To study the phosphorus content in eggshells and bones and phosphorus solubilisation efficiency by the selected bacterial inoculation, two different methods were conducted. The conditions are 0% PK broth and 25% PK broth. Each broth which added with ~1g of eggshells and ~1g of animal bones separately were then inoculated with *Rhizobium sp*. The amount phosphate released was measured for 6 days. This is to study the ability of the bacterial strain to solubilize the phosphate in the broth. Figure 22 shows the standard graph of phosphorus concentration by using stannous chloride solution.

As it can be observed in the graph (Figure 23), the amount of inorganic phosphate released from the bones and eggshells is being increased. This shows that the inoculated strain has digest and solubilise the organic P present in the bones and eggshells and released into the medium efficiently. The broth con-

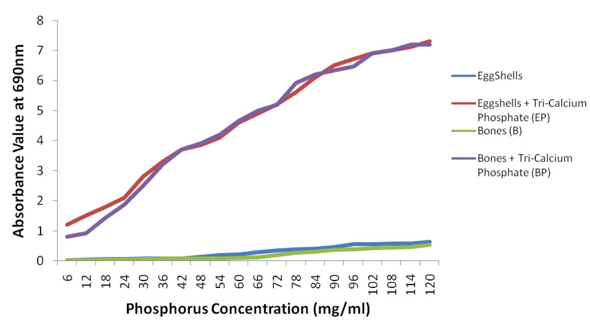

Fig. 1. Total amount of PO_4 (mg) released in 0% and 25% of PK broth

Table 6. Results of plant parameters after 55 days of seedlings

Plant Condition Parameters	Mean Shoot Length (cm)	Mean Number of leaves	Mean Number of fruits	Mean Fruit length (cm)	Mean Diameter of fruit (cm)	Mean Fruit Weight (g)	Total Chlorophyll content mg/g of leaf		
							Chl a	Chl b	Total Chlorophyll
A	22	21.12	2.33	17.33	9.5	74.66	2.711	1.024	3.735
B	23.22	23.66	2.4	20.33	9	159.71	3.021	1.127	4.148
C	23.33	22.45	2.2	18.32	7.67	198.55	2.004	1.909	3.913
D	25.11	27.11	2.5	20.25	10.83	227.34	3.484	1.073	4.557
E	26.44	25.45	2.67	17.22	9.5	116.31	2.507	1.7	4.207
F	25.67	24.78	2.8	20.75	12.6	192.94	3.659	2.273	5.932
G	27.34	26	3.1	18.55	12.2	238.09	3.393	1.821	5.214
H	31.67	29.11	3.6	23.66	13.25	297.41	3.514	2.701	6.215

taining 25% of tricalcium phosphate and eggshells together with bones showing high concentration value compared to 0% of PK broth. This indicates that the bacterial strain solubilised P efficiently with the presence of inorganic P.

Discussion

Phosphorus is an essential macronutrient that is required by the plants to enhance their plant growth. However, as mentioned earlier this condition is in critical zone in Malaysia. Since Malaysia is a heavy rainfall country, it causes the P deficiency in the soil and it affects the plants nutrients. This condition leads to the high usage of chemical fertilizers of P which subsequently increases the cost of chemical fertilizers.Therefore, this research was conducted to find an alternative way to supply a better source of insoluble, inorganic and natural P in the form of rock phosphate, bones and eggshells that can be provided together with PSB. PSB is a range of bacteria from rhizosphere of the plant to solubilise the insoluble P to soluble (Rodriguez and Fraga, 1999). In this study, the PSB tends to solubilise the rock phosphate, bones and eggshells to release P for the plant uptake and also to enhance the plant growth. The microorganisms are also known as PGPR. The PSB were isolated from rhizosphere region of *Solanum melongena* and used for further studies.

In this study, fiftythree PSB strains were isolated from three different locations and six of the strains formed clear halo zones.These strains were then purified for confirmation and the clear zones was observed. Conversely, the strain named RSH49 has been selected as high efficiency solubilising bacteria on PK media. The potential strain was then further characterized by several biochemical tests and it was identified as *Rhizobium sp.* The strains were applied to the *S.melongena*seedlings to understand the rhizosphere affects and other plant parameters were also studied. The colony (RSH49) formed high solubilising efficiency in PK media with the value of 116%. According to Zhang *et al.,* 2019 and Gupta *et al.,* 2012, a PSB tends to solubilise the P content more than 50% considered as good strain to study rhizosphere effects. The diameter of the halo zones that formed was 3.0mm to 3.5mm. This indicated that the selected strain was a good phosphate solubilising bacteria which tends to solubilise the organic P more efficiently.

Plant parameters

Plant parameters such as mean shoot length, mean number of leaves, mean number of flowers, mean number of fruits, mean length of the fruits and also mean weight of the fruits were measured as the indicator of yield.

Mean shoot length

The mean shoot length of *S.melongena*inoculated with *Rhizobium sp.*improved better when compared with the uninoculated control plants in set A, B, C and D. The highest mean shoot length of bacterial uninoculated plant was only 11.00cm. However, for the inoculated plants, the mean value was much greater, 14.00cm (Table 6). Eventually, the fertilizer application of RP and TSP in uninoculated plant doesn't bring much difference compared to the control uninoculated. Whereas, the bacterial inoculated with TSP application plant shows high mean shoot length (13.00cm) compared to rock phosphate (11.60cm). This phenomenon clearly describe that Triple superphosphate has highest P solubilisation ability than the Rock phosphate.

According to Zaharah *et al.,* (1990), TSP tends to solubilise P quickly due to the highest soluble P (98%) content that easily available in the soil. Nevertheless, RP is an insoluble form of P which solubilises P in low rate stay longer in the soil than TSP. Thus, the obtained result shows positive effects of phosphate solubilising efficiency through rhizosphere effect of PSB inoculated than the uninoculated. In addition, studies related to PGPR has reported that rhizobacteria are potential growth enhancer in different kinds of crop production such tomato, sorghum and others (Baldani *et al.,* 1986; Bashan *et al.,* 1989).

Number of leaves and flowers.

These parameters indicated that the plant growth was in good condition. The both parameters are showing higher value at bacterial inoculated plants compared to the uninoculated. The means values are raised doubly at 55 days of seedlings. Though, the valued are still high at application of bones and eggshells and followed by TSP then RP. The result clearly indicates that the PSB has elevated positive effect on the test plant.

Number, length, diameter and weight of the fruits

These parameters were performed to compare the outcome / crop production of the plant. Simultaneously, this indicates the nutrients levels that obtained by the plants from the soil through rhizosphere effect which enhance the growth factors. Interestingly, these parameters showed positive effect on bacterial inoculated plants than the uninoculated. Apparently, these parameters showed high values at the application of eggshells and bones. Based on statistical analysis, 95% confidence that the P value is <0.05. Thus, the statistical analysis is significant.

Chlorophyll Measurement

Basically, the chlorophyll content of plant leaves is an indicator which shows the plants health condition (Carlson and Simpson, 1996). Based on the obtained result, the bacterial inoculated plants are showing highest total chlorophyll compared to the uninoculated plants. The highest total chlorophyll content for uninoculated plants is 4.56mg/g whereas for inoculated is 6.22mg/g (Table 6). The both highest values are achieved by the plant of eggshells and bones application. Followed by that, the plants which applied with chemical fertilizer (TSP) showed second highest and followed by RP. So, here the clear observation can be seen that the organic fertilizer tends to produce more nutrients which increase the chlorophyll level in the leaf than the others and make the plant healthier.

Moreover, according to Shankar *et al.,* (2013) on the effect of PSB on plant growth and development of chilli and maize showed high chlorophyll content with the application of *Pseudomonas aeruginosa*. The chlorophyll content for maize was 32.89mg/g and the control was 15.61. Correspondingly, the chilli showed 27.49 mg/g of chlorophyll content over control is 18.79 mg/g. The maximum amount of chlorophyll pigment shows high rate of photosynthesis. Thus, conclusion has made that there is high uptake of P and N which contributes to direct and positive effect between PSB on soil phosphorus (Kim *et al.,* 1989).

Bone Experiment

To study the phosphate solubilizing efficiency of *Rhizobium sp.*, two different types of PK broth were prepared with two different conditions of PK broth and the amount of P released from the bones and eggshells were observed over the period of time- 7 days. Consequently, the amount of soluble phosphate from both condition 0% and 25% of PK broth shows constant increases (Figure 23). Yet, the efficiency of solubilisation is high at 25% of PK broth than the 0%.This is because, in 25% of modified PK broth, the strain has used up the presence of soluble P (Tri-calcium) due to the initial supply of P. Continually, the strain has solubilised the organic form of P (bones and eggshells) more efficiently and released into the medium. Thus, the phosphorus concentration is high at 25% of PK broth. Nonetheless, in 0% of PK modified broth the strain has solubilised the inorganic P without presence of any soluble P. So, the tendency to solubilise the inorganic P is lower compared to the other. Thus, the phosphorus concentration is lower at 0% of modified PK broth.This experiment was to prove that the *Rhizobium sp.* is an efficient Bio-fertilizer.

Overall, the results which involved of various plant parameters showed greater plant growth with the application of eggshells and bones. It's indicates that these wastes can used as partial organic and insoluble P fertilizer which available for long time period in the soil and play role in the phosphate solubilisation by rhizobacteria. Besides that, the common availability of these wastes is definitely suited for home garden. Inversely, the inorganic fertilizers such RP and TSP almost showed similar same response towards *S. melongena* seedlings.

Conclusion

In conclusion, this current study supports the theory of application of RP, bones and eggshells together with the *Rhizobium sp.* a phosphate solubilising bacteria tends to increase the crop yield of *S. melongena* by providing a better rhizosphere effect towards the plant and alternatively saving the cost from application of triple superphosphate chemical fertilizer and solve the problem of leaching effect in Malaysia climatic condition.

References

Anand, A. 2012. Agrilore-Biofertilizers Use in Tomato. Available at (http://agropedialabs.iitk.ac.in/agrilore/?q=taxonomy/term/963).

Baldani, V.L.D., Alvarez, M.AB., Baldani, J.I. and Döbereiner, J. 1986. Establishment of inoculated Azospirillum spp. in the rhizosphere and in roots of eld grown wheat and sorghum. *Plant Soil.* 90: 35-46.

Bashan, Y., Singh, M. and Levanony, H. 1989. Contribution of Azospirillumbrasilense Cd to growth of tomato seedlings is not through nitrogen xation. *Can.*

J. Bot. 67: 2429-2434

Bhatti, T. M. and Yawar, W. 2010. Bacterial solubilization of phosphorus from phosphate rock containing sulfur-mud. *Hydrometallurgy.* 103 : 54–59.

Carlson, R.E. and Simpson, J. 1996. A Coordinator's Guide to Volunteer Lake Monitoring Methods. *North American Lake Management Society*. p. 96

Carson, S. D. 1976. Ammonium molybdate-stannous chloride determination of orthophosphate in the presence of triton X-100. *Analytical Biochemistry*. 75 : 472–477. doi:10.1016/0003-2697(76)90102-0

Christiansen, W.C. 1992. N2-fixation by ammonium-excreting *Azospirillum brasilense*in auxin-induced tumours of wheat (*Triticum aestivum* L.). *Biol. Fertil. Soils.* 12 : 85–100.

Cornelissen, J., Lavorel, S., Garnier, E., Diaz, S., Buchman, N., Gurvich, D., Reich, P., Steege, H., Morgan, H., Heijden, M., Pausas, J. and Poorter, H. 2003. A protocol of standardized and easy measurement of plant functional of traits worldwide. *Australian Journal of Botany.* 51 : 335-380.

Fernandez, C. and Novo, R. 1988. Vida Microbianaen el Suelo. *Universidad de La Habana*. 525.

Glick, B.R. 1995. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol*. 41 : 109–17.

Goldstein, A.H. 1994. Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by gram-negative bacteria. In: Torriani-Gorini A, Yagil E, Silver, S, editors. Phosphate in Mi- croorganisms: *Cellular and Molecular Biology*. pp. 197–203.

Goldstein, A.H. 1986. Bacterial solubilization of mineral phosphates: historical perspective and future prospects. *Am. J. Altern Agri.* 1 : 51–7.

Goldstein, A.H. and Liu, S.T. 1987. Molecular cloning and regulation of a mineral phosphate solubilizing gene from Erminia Herbicola. *Nature Biotech.* 5 : 72-74.

Gupta, M., Kiran, S., Gulati, A., Singh, B. and Tewari, R. 2012. Isolation and identification of phosphate solubilizing bacteria able to enhance the growth and aloin-A biosynthesis of Aloe barbadensis Miller. *Microbiological Research.* 167 : 358–363. doi:10.1016/j.micres.2012.02.004

Jones, D.A., Smith, B.F.L., Wilson, M.J and Goodman, B.A. 1991. Solubilizator fungi of phosphate in rise soil. *Mycol. Res.* 95 : 1090–1093.

Kim, K.Y., Jordan, D. and McDonald, G.A. 1989. Effect of phosphate-solubilizing bacteria (PSB) and VAM on tomato growth and soil microbial activities. *Biology of Fertility Soils*. 26: 79-87

Kuttner, T. and Lichtenstein, L. 1930. Micro colorimetric studies: ii. Estimation of phosphorus: molybdic acid-stannous chloride reagent. *J. Biol. Chem.* 86 : 671-676.

Leong, J.S. 1986. Their biochemistry and possible role in the biocontrol of plant pathogens. *Annu. Rev. Phytopathol*. 24 : 187–208.

Rodriguez, H. and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* 17 : 319-339.

Shankar, T., Sivakumar, T., Asha, G., Sankaralingam and Meenakshi, S. 2013. Effect of PSB on Growth and Development of Chilli and Maize Plants. *World Applied Sciences Journal.* 26 : 610-617.

Sims, J.T. and Sharpley, A.N. (ed.) 2005. *Phosphorus: Agriculture and the Environment, American*. Madison, Wisconsin, USA Publishers.

Sivan, A. and Chet, I. 1992. Microbial control of plant diseases. In: Mitchell R, editor. Environmental Microbiology.Society of Agronomy, strain. *Acta Biotechnol*. 21 : 359–369.

Sood, R. 2006. *Textbook of Medical Laboratory Technology*, 1st edition.India: Jaypee Brothers Mediacal Publishers (P) Ltd.

Trivedy, R.K. and Goel, P.K. 1986. Chemical and Biological Methods for Water Pollution Studies, *Environmental Publications*, Karad, India

Willey, J., Sherwood, L. and Woolverton, C. 2008. *Prescott, Harley and Klein's Microbiology*, 7th ed. Boston : Mc Graw-Hill

Xie, H., Pasternak, J. J. and Glick, B.R. 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. *Curr. Microbiol*. 32 : 67–71.

Zaharah, A.R., Hawa, J. and Sharifuddin, H.A.H. 1990. Initial and Residual Value of P from Apetite Rock Phosphates and Superphosphates as Measured by ^{32}P Dilution Technique. *Pertanika*. 13 : 27-31.

Zaharah, A.R., Hawa, J. and Sharifuddin, H.A.H. 1985. Accumulation and Migration of Phosphate Applied as Rock Phosphate in an Oil Palm Plantation. *Pertanika*. 8 : 317-321.

Zhang, T., Hu, F. and Ma, L. 2019. Phosphate-solubilizing bacteria from safflower rhizosphere and their effect on seedling growth. *Open Life Sci*. 14: 246–254.