Determination of Desirable Properties of Bacteria, Fungi and Their Biofilm Associated with Rubber Rhizosphere

R.P. Hettiarachchi[*], R.S. Dharmakeerthi[1], G. Seneviratne[2], A.N. Jayakody[1], K.E. De Silva T. Gunathilake and A. Thewarapperuma

Rubber Research Institute of Sri Lanka
Dartonfield
Agalawatta

ABSTRACT: *Rhizosphere microbes and their community structure 'biofilm' play a significant role in maintaining the dynamic of soil fertility. In this study, isolated bacteria and fungi from rubber root rhizosphere in red yellow podzolic soils and their biofilm structures were formulated under laboratory conditions. They were evaluated for their effectiveness on solubilization of insoluble inorganic compounds, calcium hydrogen phosphate ($CaHPO_4$), higher grade Eppawala rock phosphate (HERP) and Eppawala rock phosphate (ERP)) in liquid medium, production of indole acetic acids (IAA) and capacity of fixing atmospheric nitrogen using acetylene reduction assay (ARA). The relationships between variables were identified using regression analysis. The solubilization of $CaHPO_4$ in liquid medium by different bacterial strains was accompanied by a drop in pH (4.2-6.1) from an initial pH of 6.8-7.0, which followed a polynomial relationship between the pH of the medium and the amount of soluble phosphorus. The medium with HERP showed less relationship compared to $CaHPO_4$ between the above parameters. No correlation was found between pH and the amount of P solubilized of the culture medium containing ERP. Out of 30 bacterial isolates, five isolates formed proper biofilm community structure. The biofilm solubilized significantly high amount of phosphorus in liquid medium containing $CaHPO_4$ compared to their bacterial and fungal counterparts and was observed synergistic effect for ARA. Production of IAA of biofilm was higher than that of bacteria alone cultures. Thus the biofilm formation of rhizosphere microbes seems to be very important for improved soil fertility.*

Key words*: Acetylene reduction assay, indole acetic acids, phosphorus solubilization, rhizosphere microbes*

INTRODUCTION

Rubber plantations were first established in Sri Lanka at the beginning of the 20th century. Since then, many individual plantations have undergone 3 or 4 planting cycles. Depleted soil nutrients must be replenished through balanced and efficient organic and inorganic fertilizers and through improved soil management practices. Among the organic sources, biofertlizers have been recognized as economical alternatives for imported fertilizers.

[1] Department of Soil Science, Faculty of Agriculture, University of Peradeniya, Peradeniya
[2] Institute of Fundamental Studies, Hanthana road, Kandy
[*] Corresponding author e-mail: *rasikarri@yahoo.com*

Soil is an excellent niche of microorganisms, *i.e.* protozoa, fungi, bacteria and viruses. Some soil bacteria that are able to colonize surrounding the plant roots is called rhizobacteria, and play a significant role in maintaining the dynamic of soil fertility and plant growth (Kloepper, 2003; Bakker *et al.*, 2007). Rhizobacteria which provides beneficial effects on the host plant growth *via* direct and indirect mechanisms, are referred to as plant growth promoting bacteria (PGPB) (Kloepper and Schrot, 1981). These bacteria support fixation of atmospheric nitrogen, solubilization of phosphatic minerals and secretion of stimulating hormones, like auxin also known as Indole 3 Acetic Acid (IAA) (Huddedar *et al.*, 2002; Chopade *et al.*, 2008; Bashan and de-Bashan, 2010). Nitrogen fixing capabilities of these strains had been quantified using the nitrogenase enzyme, which reduces acetylene to ethylene, and the production of ethylene was measured using gas chromatography (Dilworth, 1966; Stewart *et al.*, 1967; Staal *et al.*, 2001). Solubilization of insoluble inorganic aluminum and iron phosphate compounds is an important strategy for increasing availability of phosphorus in the soil (Barber, 1984; Tan, 1993; Marschner, 1995). Some soil bacterial isolates have an ability to solubilize inorganic low soluble phosphates (Illmer *et al.*, 1995; Wakelin *et al.*, 2004; Chen *et al.*, 2006; Song *et al.*, 2008).

Solubilization of insoluble inorganic compounds ($CaHPO_4$, HERP & ERP) in solid and liquid medium has been widely used as the isolation methodology of phosphate solubilizing microorganisms (Pikovaskaya, 1948). Plant growth promoting rhizobacteria can also promote plant growth through secretion of plant growth regulators; e.g. IAA (Lee *et al.*, 2004) and can be quantified by following the protocol adapted by Gordon and Weber, 1951. Isolation of indigenous microbes capable of solubilizing phosphorus, fixing of atmospheric nitrogen and secretion of plant growth promoting hormones is an important procedure because of their superior adaptability to the isolated environment than the introduced strains.

Community of microbes consists of microbial cells with extracellular biopolymers are named as a biofilm (Seneviratne, 2003 and Seneviratne *et al.*, 2008b). Further, biofilms are more efficient than their monocultures or mixed cultures of effective microbes and studies so far gave encouraging results on soil fertility (Seneviratne *et al.*, 2008, 2009 and 2011). Considerable attention has therefore been focused recently on microbial interference on biofilm formation in the environment and their potential to increase nutrient availabilities in the soil.

The objective of this study were to (i) measure the phosphate solubilizing, nitrogen fixing and IAA production capacities of some indigenous bacterial fungal species isolated from rhizosphere of Rubber plants (*Hevea brasiliensis*), (ii) select promising strains for the formation of biofilm and evaluate their effectiveness under *in-vitro* conditions.

MATERIALS AND METHODS

Isolation of indigenous rhyzosphere micro organisms

Root samples were collected from a mature rubber field at Dartonfield Estate, Agalawatta. They were placed on petri dishes containing PDA and morphologically distinct fungal colonies were identified and sub-cultured on same medium to purify. Root samples were shaken in 50 ml sterilized distilled water for 30 minutes to extract rhizosphere bacteria. Samples were then serially diluted in sterilized distilled water and suspensions were spread on nutrient agar (NA) plates. Cultures were incubated at $28 \pm 2°C$ for 24 to 48 hours.

Morphologically different colonies were isolated by sub culturing of single colonies on NA plates (Parkinson *et al*., 1971).

Screening of efficient bacterial strains

Qualitative estimation of phosphorus solubilization

Bacterial cultures were grown in NA broth using $10^8 - 10^9$ cells ml^{-1} colony forming unit (cfu) were inoculating onto Pikovaskaya (PYK) plates amended with 0.4% calcium hydrogen phosphate ($CaHPO_4$). After 2-3 days, the clear zone appeared around the isolates was observed.

Quantitative estimation of phosphorus solubilization

Thirty bacterial isolates were grown in NA broths were transferred into 20 ml of PYK broth supplemented with 0.4 % of $CaHPO_4$. They were incubated on a rotary shaker (60 rpm) with continuous shaking at 28 ± 2°C for 6 days. Each incubated broth cultures with four replicates were sampled periodically at one day interval and the supernatant of the culture was separated after centrifuging at 6000 rpm for 15 minutes. Quantitative analysis of phosphorus was done by the molybdenum-blue method (Murphy and Riley, 1962) with Skalar Auto Analyzer and pH of supernatant was determined using Jenway pH meter with glass electrode. Based on the clustering pattern of P solubilization, isolates which were unable to solubilize more P (less than 100ppm) sorted out under one cluster and those isolates were not further evaluated. Twenty two bacterial isolates that resulted high P solubilization (higher than 100ppm) were grouped under two clusters were further assessed for their P solubilization with HERP and ERP. Rock phosphates (RP) were obtained from a locally available RP deposit at Eppawala (ERP), which has 38 % and 30% of total P_2O_5 in HERP and ERP, respectively.

Quantification of indole acetic acid (IAA) production

The second screenings of isolated bacterial cultures were done on the basis of IAA production. Selected twenty two single bacterial cultures were incubated for 14 days in Tris-YMRT medium and centrifuged at 3000 rpm for 15 minutes. One milliliter aliquot of the supernatant was vigorously mixed with 4ml of Salkowski's reagent. Absorbance of each culture medium was measured at 535 nm and concentrations were determined with the calibration curve prepared with analytical grade indole-3-acetic acid (Gordon and Weber, 1951).

Acetylene reduction assay (ARA) of bacteria

The twenty two isolates were further characterized on their ability of nitrogen fixation or acetylene reduction assay. The starting medium for acetylene reduction assay was the semi-solid glucose + malate medium as described by Patriquin *et al*. (1980). This medium was modified to optimize ethylene production and was then named ML medium (Penido *et al*., 1985; Park *et al*., 2005). Autoclaved medium was cooled to 50°C and 4ml of aliquots were transformed into 18 ml vials. Flasks containing 20 ml of combine carbon medium (CCM) broth were inoculated with 10 µl of a broth culture of each bacteria in late lag phase, and incubated for maximum growth at 32°C with mild shaking. Each of these cultures containing 70 µl were then transferred separately to the ARA assay vials, which containing 4 ml of ML

medium. These vials were incubated aerobically, for 24 hours at 32°C. After incubation, cotton plugs were replaced with a rubber stopper and 1.8ml of acetylene was injected in to each sample. After incubation for 1 hour at 30°C, 0.3 ml of sample from the gas was withdrawn and injected to a gas chromatography (Porapak N column at 110°C and N_2 as the carrier gas at a flux of 120 ml/minute) (Rodrigues *et al.*, 2008).

Effectiveness of biofilms

Selected twenty two bacterial isolates and most common fungi *Aspergillus* were maintained in yeast manitol broth (YMB) separately. They were co-cultured for the formation of biofilm according to the methodology developed by Seneviratne *et al.* (2006). They were evaluated for their effectiveness on P solubilization in broth medium with 1% of $CaHPO_4$, acetylene reduction assay and indole acetic acid production.

Statistical analysis

Mean values and standard deviations were calculated to represent P solubilization with $CaHPO_4$. Moreover, hierarchical clustering analysis was done employing the statistical package GenStat 17 to categorize the isolates based on their P solubilization with $CaHPO_4$. Regression analysis were came out to derive the relationship between solubilized phosphorus and their growth medium acidity with three different P sources $CaHPO_4$, HERP and ERP. Effectiveness of phosphorus solubilization in biofilm was observed using two orthogonal comparisons (a) bacterial cultures with fungal only and biofilm (b) fungal only cultures with biofilm. Analysis of variance was done to compare nitrogenase activity (ARA) and Indole acitic acid (IAA) production in between bacteria and their biofilm community.

RESULTS

Qualitative estimation of phosphorus solubilization

Out of 30 bacterial isolates, ten isolates were able to solubilize $CaHPO_4$ and resulted halos on $CaHPO_4$ inoculated PYK solid medium (Figure 1a), whilst other bacteria did not produce solubilization halo around their colonies (figure 1b).
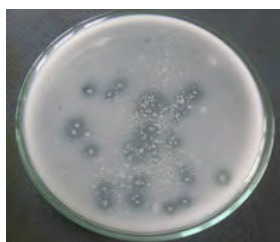

Fig. 1(a)   Fig. 1 (b)

Fig. 1.   Solubilization of low soluble inorganic phosphate ($CaHPO_4$) on PYK solid medium was observed by halos produced by bacterial isolates (a). Solubilization halo non-producing bacterial isolates (b).

Quantitative estimation of phosphorus solubilization

In liquid medium with $CaHPO_4$, out of 30 bacterial isolates, 22 were capable of solubilizing phosphorus higher than 100ppm (Table 1) and were clustered in two different regions (Figure 2). The isolates which were able to solubilize phosphorus less than 100ppm were clustered separate to others (Figure 2). Bacterial isolates those have an ability to solubilize phosphorus more than 100ppm of the medium with $CaHPO_4$ were further evaluated for their phosphorus solubilization of the medium with HERP and ERP. For the medium with HERP, the strains coded as B3, B5, B6, B7, B9, B17, B23 and B25 increased the soluble phosphorus content more than 20 ppm at the end of the incubation period (mean value = 33.55ppm). In the medium containing ERP, 5 strains coded as B5, B6, B17, B23 and B25 out of 22 strains increased the soluble phosphorus content more than 20ppm at the end of the incubation period (mean value = 34.1ppm). In general, strains B5, B6, B17, B23 and B25 exhibited higher solubilizing capability for the three sparingly soluble inorganic phosphates in liquid medium than the other strains and it was evidenced by the clustering pattern of the dendrogram based on phosphorus solubilization in the medium with $CaHPO_4$ (Figure 2).

Table 1. Mean soluble P contents by different bacterial isolates

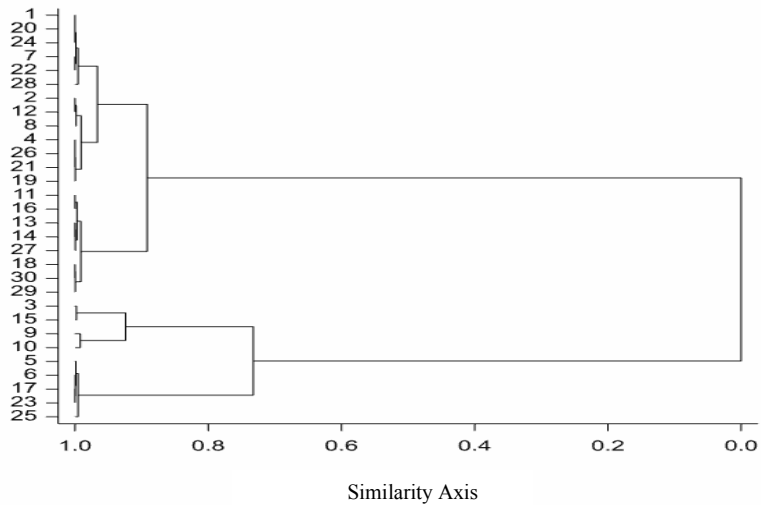| Isolate No.	Mean soluble P with $CaHPO_4$/ppm
1B	123 ±10.8
2B	150 ±7.5
3B	263 ±15.6
4B	133 ±8.1
5B	307.6 ±11.2
6B	317.6 ±2.5
7B	116 ±7.0
8B	142 ±22.9
9B	192 ±9.0
10B	215 ±7.0
11B	89 ±10.3
12B	153 ±7.6
13B	80 ±10.2
14B	79 ±4.7
16B	93 ±4.2
17B	313 ±5.9
18B	69 ±4.6
19B	128 ±11.7
20B	122 ±14.2
21B	133 ±9.1
22B	114 ±0.6
23B	312 ±2.1
24B	124 ±11.4
25B	325 ±10.5
26B	133 ±4.2
27B	84 ±7.2
28B	106 ±7.1
29B	74 ±14.0
30B	70 ±5.0

Fig. 2. **Dendrogram for solubilization of phosphorus by different bacterial strains in liquid medium with CaHPO$_4$.**

Quantitative estimation of phosphorus solubilization with biofilm

Out of 30 bacterial isolates, 14 bacterial isolates had an ability to colonize on fungal mycelia and formed biofilm community structure (Figure 3). The rest did not form biofilm.
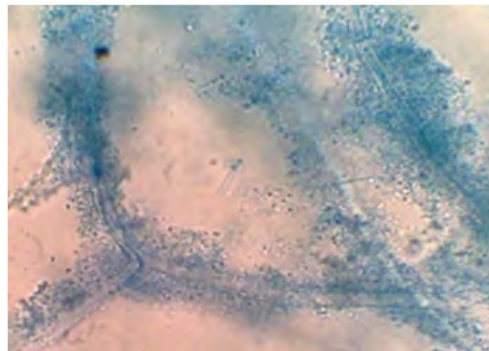

Fig. 3. **A microscopic view of fungal filaments attached by bacteria for the formation of fungal bacterial biofilm Magnification: 2,000 x.**

Fast growing fungi formed mat like structure when they were grown on liquid culture medium. The fungi those were co-cultured with bacteria for the formation of biofilm was also observed the same mat structure on the top of the medium. Part of the soluble phosphorus in liquid culture medium, produced by fungal alone culture or biofilm could be deposited in that mat like structure. In order to evaluate the effectiveness of phosphorus solubilization in biofilm, orthogonal comparison was made to compare bacterial broth

containing soluble P (variable 1) with fungi alone (variable 2) and their biofilm culture (variable 3) (Table 2). This contrast effect was significant at the probability level p< 0.0001.

Table 2. Sparingly soluble phosphorus sources solubilized by different microbial treatments after 07 days of incubation

Treatment	$CaHPO_4$ solubilized (ppm)
Bacteria only (B25)	325.3[c]
Fungi only (F2)	6660[b]
Biofilm (B25F2)	9600[a]

Phosphorus solubilization and growth medium acidity (pH)

The majority of bacterial strains acidified liquid medium containing three insoluble inorganic phosphates. The solubilization of $CaHPO_4$ in the liquid medium by different strains was accompanied by a drop in pH (4.2 to 6.1) from an initial pH of 6.8-7.0 after the incubation period of 72 hours. There was an inverse correlation between the pH of the medium and the released of P content in the supernatant of the culture medium containing $CaHPO_4$ and HERP as a sole source (Figure 4a and 4b). No correlation was found between pH and the amount of P released in the culture medium containing ERP (Figure5).
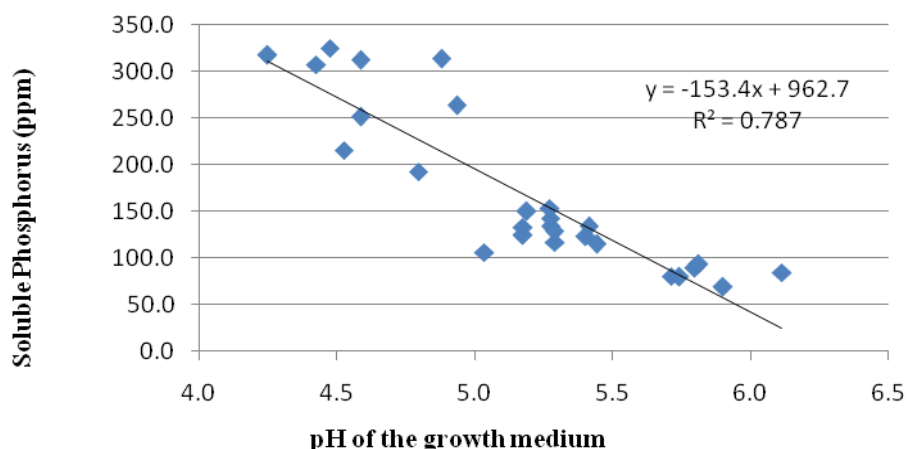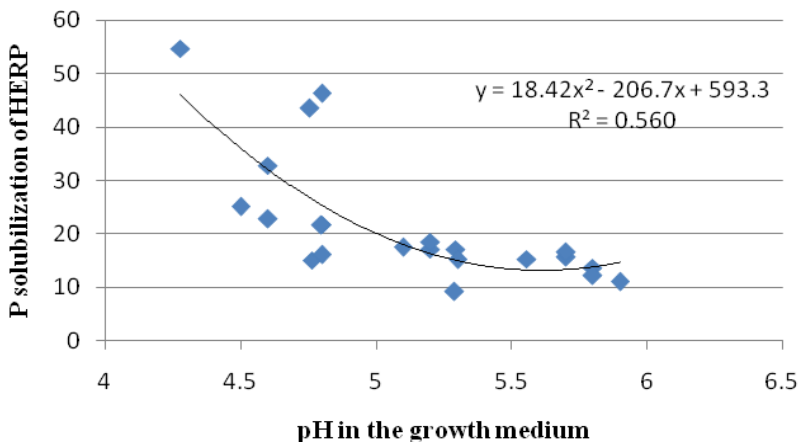

Fig. 4 a

Fig. 4 b

Fig. 4 a. & 4 b. **Relationship between pH and solubilized phosphorus by bacterial strains in liquid medium with CaHPO$_4$ (a) and HERP (b).**
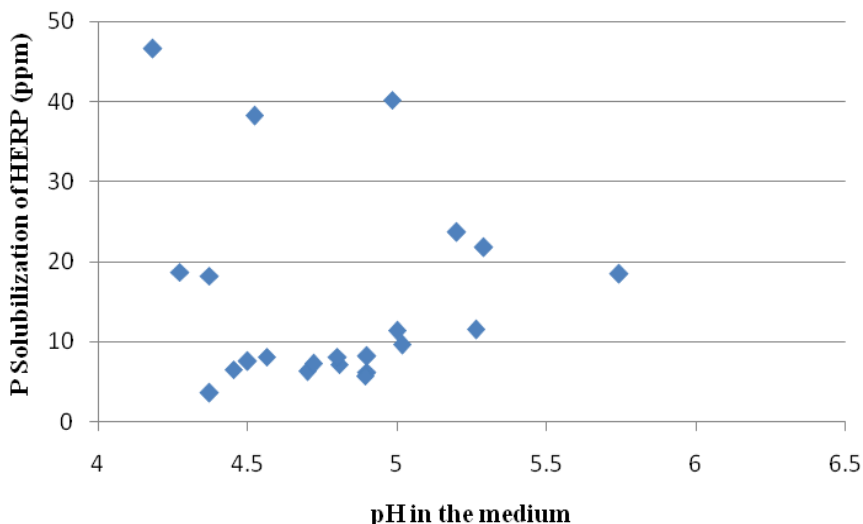

Fig. 5. **Relationship between pH and solubilized phosphorus by bacterial strains in liquid medium with ERP**

Indole acetic acid production (IAA) of isolates

Out of 22 bacterial isolates which are capable of CaHPO$_4$ solubilizing higher 100ppm, 9 isolates were screened based on indole acetic acids (IAA) production ability. Those isolates were coded as B2, B3, B4, B6, B7, B12, B19, B23 and B25. There were significant differences (P<0.0001) among the nine isolates on the production of IAA which ranged from 0.3-4.8μg/ml, with mean value of 3.0122 ± 1.26. Same pattern was observed on the production of IAA among the biofilm community structures and it ranged 0.7-7.9 mg/ml, with the mean value of 4.9348 ± 1.98. Furthermore, a significant difference ((P<0.0001) in the production of IAA was recorded in the presence of particular biofilm community

structures (B2F2, B3F2, B6F2, B23F2, B25F2) compared to their bacteria alone counterpart (Figure 6).
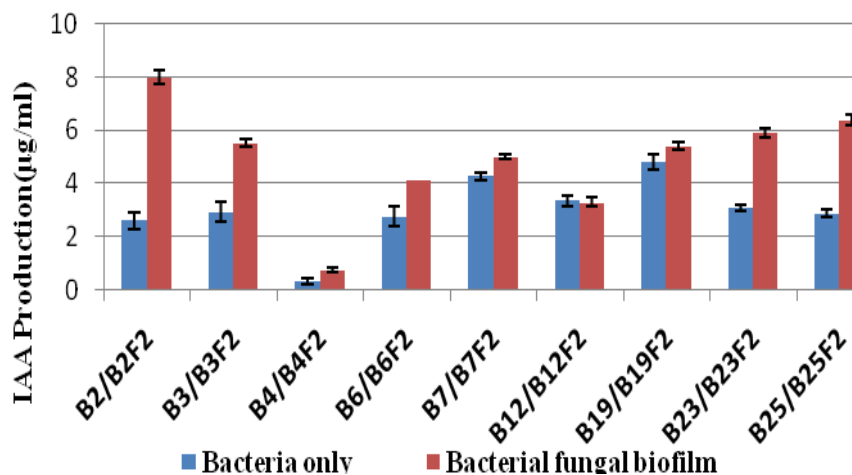

Fig. 6. Indole acetic acid production of medium containing different bacteria and their biofilm community structures

Nitrogenase activity was observed with 3 bacterial isolates which were coded as B3, B19 and B25 out of total 30 bacterial isolates and it was ranged from 3-8 μmol C_2H_4/hr. Moreover, some bacterial isolates that exhibited either no or low nitogenase activity, showed significantly ($p<0.0001$) higher nitrogenase activity with their biofilm community structure (Table 3).

Table3. Comparison of nitrogenase activity (μmol C_2H_4/hr) in between bacteria and their biofilm community

Code of Bacteria and their biofilm	Difference of nitrogenase activity (μmol C_2H_4/hr) between bacteria and their biofilm
B6 and B6F2	90.65[a]
B23and B23F2	82.84[a]
B25and B25F2	40.74[b]
B12and B12F2	8.67[c]

DISCUSSION

Most of the selected morphologically different bacterial isolates showed rapid growth on culture medium. Phosphorus solubilization in solid medium was observed from the production of halo with some bacterial isolates in the media containing $CaHPO_4$. However, there were no any solubilization halos in solid medium containing HERP and ERP sources. Similar observations with $CaHPO_4$ and $Al(H_2PO_4)_3$ and $FePO_4.2H_2O$ had been reported (Marra *et al*., 2012; Ogut *et al.,* 2010 and Qin *et al.*, 2011). According to these studies, halos

appeared in sparsely soluble $CaHPO_4$ and no halos appeared in water insoluble Al and $FePO_4$. With liquid medium, all strains were capable of solubilizing $CaHPO_4$, HERP and ERP with varying degrees. Many of the strains which did not display a solubilization halo in solid medium were capable of solubilizing phosphorus in liquid medium. All isolated bacterial strains were capable of solubilizing $CaHPO_4$ in liquid medium and similar results were observed in a study evaluating the solubility of $CaHPO_4$ in liquid PYK medium by bacteria from the rhizosphere of *Allium fistolusum* L., *Capsicum annum* L., *Sesamum indicum* L., and *Oryza sativa* L. (Chung *et al*., 2005). Lower solubilization index with solid medium without formation of solubilization halo around the bacterial isolates was promoted in liquid medium with favorable conditions for the diffusion of organic acids (Marra *et al*., 2012). Biological, chemical and physical properties may engage with the ability of soil microorganisms to solubilize insoluble inorganic phosphate. In many cases, acidification is the key process involved in phosphate solubilization. A significant negative correlation between the pH of the culture medium and phosphorus solubilization by several genera and species were observed by many authors (Illmer and Schinner, 1995; Chen *et al*., 2006; Marra *et al*., 2011). There was an inverse correlation between pH of the liquid culture medium and the released of P content in the supernatant of the culture medium containing $CaHPO_4$ and HERP as a sole sources. The organic acids released by phosphorus solubilizing bacteria could drop pH of the growth medium (Maliha *et al*., 2004; Pradhan & Sukla, 2005). No correlation was found between pH and the amount of P solubilized of the culture medium containing ERP. The reduction of pH in some cases does not correlate with solubilized phosphorus (Chen *et al*., 2006; Khan *et al*., 2007b). Alternative possibilities other than organic acids for inorganic P solubilization have been reported. For example, extrusion of proton accompanied with the cation uptake (Khan *et al*., 2007b), formation of exopolysaccharide (Yi *et al*., 2008), production of chelating substances (Whitelaw, 2002; Kucey, 1988; Welch *et al*., 2002) and through exchange reactions (Gahoonia *et al.,* 1992; Jones, 1998; Trolove *et al*., 2003) can be highlighted. Present study indicated that, the formation of biofilm with effective microbes has an ability to solubilized sparingly soluble $CaHPO_4$ more than their bacterial and fungal counterparts. Similar observation has been made by the others (Seneviratne and Indrasena, 2006); Jayasinghearachchi and Seneviratne, 2006) where 8-13 fold increment of phosphorus solubilization was observed with biofilm application. This could be due to high level of organic acids production associated with biofilm community with high level of gene expression and it was different from their individual microbes present in the environment (Vilain and Brozel, 2006).

Some bacterial isolates B2, B3, B6, B7, B12, B17, B23 and B25 the secreted IAA ranging from 2.5-5ppm than the other bacterial isolates. Moreover, the IAA production of biofilm was ranging from 3-7ppm and higher than their mono cultures. There is evidence that the capacity of producing IAA was higher with biofilm compared to their bacterial only cultures (Bandara *et al*., 2006; Seneviratne *et al*., 2009).

Most of the bacterial isolates associated with rubber root rhizosphere had the ability to grow in nitrogen free medium. But three bacterial isolates coded B3, B19 and B25 showed high nitrogenase activity compared to other isolates. The nitrogenase activity of the biofilms was significantly higher than that of their monocultures (Seneviratne and Jayasinghearachchi, 2005; Seneviratne *et al*., 2008). Several studies have reported the benefits of nitrogen fixing prokaryotes in growth and nutrition of many plant species; According to the observation of Jayasinghe *et al*. (1989) and Jayasinghe and Wettasinghe (1991), the nitrogen fixing ability of leguminous cover crop in rubber plantations was severely affected with high nitrogen level of the environment where the leguminous counterpart in rubber plantations is fertilized with high nitrogen fertilizers. However, the ability of the rhizosphere

microorganisms of rubber tree to fix N under such conditions had not been investigated. Research should be done in the future in order to evaluate whether the biofilms could overcome failures, if any, associated with rubber root rhizosphere, especially for nitrogen fixing capability.

The isolation and screening of IAA producing, nitrogen fixing and P solubilizing bacterial isolates associated with rubber root rhizosphere is important for the development of biotechnological product that can be used by growers as partial substitute for costly inorganic fertilizers.

CONCLUSION

We were able to isolate 14 rhizobactera spp. (coded as B1, B2, B3, B4, B6, B7, B9, B10, B12, B19, B20, B21, B23, B25) associated with rubber root rhizosphere that are not only capable of P solubilization, N fixation and IAA secretion, but also form biofilms with some *Aspergillus* sp isolated from rubber rhizosphere. It was revealed that the fungal bacteria biofilm communities are more effective in their biological performances than their monoculture counterparts.

ACKNOWLEDGEMENT

REFERENCES

Bakker, P.A.H.M., Pieterse, C.M.J. and Van Loon, L.C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology. *97*, 239 - 243.

Bandara, W.M.M.S., Seneviratne, G. and Kulasooriya, S.A. (2006). Interactions among endophytic bacteria and fungi: effects and potentials. J.Biosci. *31,* 645 - 650.

Barber, S.A. (1984). Soil nutrient bioavailability. John Wiley. New York, USA.

Bashan, Y. and De-Bashan, L.E. (2010). How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. Adv. Agron. *108,* 77 - 136.

Chen, Y.P., Reka, P.D., Arun, A.B., Shen, F.T., Lai, W.A. and Young, C.C. (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Applied Soil Ecology. *34,* 33 - 41.

Chopade, B.A., Huddedar, S.B., Shete, A.M., Tilekar, J.N., Gore, S.D. and Dhavale D.D. (2008). Plasmid encoding IAA and method thereof. United Sates Patent. *7*, 341 - 868.

Chung, H., Park, M., Madhaiyan, M., Seshadri, S., Song, J., Cho, H. and Sa, T. (2005). Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plant of Korea. Soil Biol. Biochem. *37*, 1970 - 1974.

Dilworth, M.J. (1966). Acetylene reduction by nitrogen fixing preparations from *Clostridium pasteurianum*. Biochimica Acta. *127,* 285 - 294.

Gahoonia, T.S., Claassen, N. and Albrecht, J. (1992). Mobilization of phosphate in different soils by ryegrass supplied with ammonium or nitrate. Plant and Soil. *140*, 241 - 248.

Gordon, S.A. and Weber, R.P. (1951). Colorimetric estimation of indoleacitic acid. Plant Physiology. *26,* 192 - 195.

Halder, A.K., Mishar, A.K., Bhattacharyya, P. and Chakrabartty, P.K. (1990). Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. J. gen. Appl. Microbiol. *36*, 81 - 92.

Huddedar, S.B., Shete, A.M., Tilekar, J.N., Gore, S.D., Dhavale, D.D. and Chopade, B.A. (2002). Isolation, characterization and plasmid pUPI126 mediated indole 3acetic acid (IAA) productions in *Acinetobacter* strains from rhizosphere of wheat. App. Biochem. Biotech. *102-103,* 21 - 29.

Illmer, P. and Schinner, F. (1995). Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biol. Biochem. *27,* 257 - 263.

Illmer, P., Barbato, A. and Schinner, F. (1995). Solubilization of hardly soluble AIPO4 with P-solubilizing microorganisms. Soil Biol. Biochem. *27,* 265 - 270.

Jayasinghe, C.K. and Wettasinghe, D.S. (1991). Effects of combined nitrogen on growth and nodule function of *Pueraria Phaseoloides*. Journal Rubber Research Institute of Sri Lanka. *71,* 7 - 20.

Jayasinghe, C.K., Parker, C.A., Kinhy, D.K. and Kulasooriya, S.A. (1989). Nodulation, nitrogenase activity and major nutrient constituents of common cover legumes during early growth. Journal Rubber Research Institute of Sri Lanka. *69,* 27 - 35.

Jayasinghearachchi, H.S. and Seneviratne, G. (2006b). Fungal solubilization of rock phosphate is enhanced by forming fungal- rhizobia biofilm. Soil Biol. Biochem. *38*, 405-408.

Jones, D.L. (1998). Organic acids in rhizosphere. A critical review. Plant Soil. *205,* 25 - 44.

Khan, M.S., Zaidi, A. and Wani, P.A. (2007). Role of phosphate solubilizing microorganisms in sustainable agriculture. A review Agron. Sustain. Dev. *27*, 29 - 43.

Kloepper, J.W. and Schroth, M.N. (1981). Plant growth promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathology. *71*, 642 - 644.

Kucey, R.M.N. (1988). Effect of *Penicillium bilail* on the solubility and uptake of micronutrients from soil by wheat. Can J Soil Sci. *68,*261 - 270.

Lee, S., Flores-Encarnacion, M., Contreras-Zentella, M. Garcia-Flores, L., Escamilla, J .E. and Kennedy, C. (2004). Indole-3-acitic acid biosynthesis is deficient in *Gluconacetobacter diazotrophicus* strains with mutations in *Cytochromec* biogenesis genes. J. Bacteriol. *186*, 5384 - 5391.

Marra, L.M., Soares, C.R.F.S., De Oliveira, S.M., Ferreira, P.A.A., Soares, B.L., Carvalho, R.F., De Lima, J.M. and De S. Moreira, F.M. (2012). Biological nitrogen fixation and phosphate solubilization by bacteria isolated from tropical soils. Plant Soil. *357*, 289 - 307.

Marra, L.M., Soares, C.R.F.S., De Oliveira, S.M.F.L., Oliveira, R.F., Carvalho, J.M., Lima D. and Moreira, F.M.De S. (2015). Initial pH of medium affects organic acids production but do not affect phosphate solubilization. Braz. J. Microbiol. *46(2)*.

Marschner. H. (1995). Mineral nutrition of higher plants, 2nd edn. Academic, London.

Murphy, J. and Relay, J.P.A. (1962). Modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta. *27,* 31 - 36.

Ogut, M., Er, F. and Kandemir, N. (2010). Phosphate solubilization potential of soil *Acinetobactor* strains. Biol. Fertil. Soils. *46,* 707 - 715.

Park, M., Kim, C., Yang, J., Lee, H., Shin, W., Kim, S. and Sa, T. (2005). Isolation and characterization of dizotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. Microbial Research. *16*, 127 - 133.

Parkinson, D., Gray, T.R.G and Williams, S.T. (1971). Isolation of microorganism: In: methods for studying the ecology of microorganisms. IBP Handbook. *19*, 36 - 55.

Patriquin, D.G., Gracioli, L.A. and Ruschel, A.P. (1980). Nitrogenase activity of sugar cane propagated from stem cuttings in sterile vermiculite. Soil Biology and Biochemistry. *12*, 413 - 417.

Penido, G.C.E., Elsas, J.D. and Monica, M.L. (1985). An improved method of detect acetylene reducing activity in Bacillus strains. Journal of Microbiological Methods. *3*, 147 - 157.

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil connection with the vital activity of some microbial species. Microbiologiya. *17*, 362 - 370.

Qin, L., Jiang, H., Tiao, J., Zhao, J. and Liao, H. (2011). Rhizobia enhance acquisition of phosphorus from different sources by soybean plants. Plant Soil. doi: 10,1007/s 111o4-001-0947-z.

Reddy, M.S., Kumar. S. and Babita, K. (2002). Biosolubilization of poorly soluble rock phosphates by *Aspergillus tubingensis* and *Aspergillus niger*. Bioresource Technology. *84*, 187-189.

Rodrigues, E.P., Rodrigues, L.S., DeOliveira, A.L.M., Baldani, V.L.D., Teixeira, K.R.D. Urquiaga, S. and Reis, V.M. (2008). <u>Azospirillum amszonense</u> inoculation: effects on growth, yield and N2 fixation of rice (*Oryza sativa* L.). Plant and Soil. *302,* 249 - 261.

Seneviratne, G. (2003). Development of eco-friendly beneficial microbial biofilms. Curr. Sci. *85*, 1395 - 1396.

Seneviratne, G. and Indrasena, I.K. (2006). Nitrogen fixation in lichens is important for improved rock weathering. J. Biocsi. *31*, 39 - 643.

Seneviratne, G. and Jayasinghearachchi, H.S. (2005). A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil. Soil Biol. Biochem. *37*, 1975 - 1978.

Seneviratne, G., Zavahir, J. S., Bandara, W.M.M.S. and Weerasekara, M.L.M.A.W. (2008a). Fungal-bacterial biofilms: Their development for novel biotechnological applications. World J.Microbiol.Biotechnol. *24*, 739 - 743.

Seneviratne, G., Jayasekara, A.P.D.A., Silva, M.S.D.L.De. and Abeysekara, U.P. (2011). Developed microbial biofilms can restore deteriorated conventional agricultural soils. J. Soil Biology and biochemistry. *43*, 1059 - 1062.

Seneviratne, G., Kecskes, M.L. and kennedy, L.R. (2008b). Biofilmed biofertilizers: novel inoculants for efficient nutrient use in plants in Proceedings of a Project (SMCN/2002/073) Workshop Held in Hanoi, Vietnam, 12-13 October 2007. pp. 126-130. In: Kennedy, L.R., Choudhury, A.T.M.A., Kecskes, M.L. and Rose, M.L. (Ed.) Efficient nutrient use in Rice production in Vietnam Achieved Using Inoculant Biofertlizers.

Seneviratne, G., Tennakoon, N.S. and Weerasekara, M.L.M.A.W. (2006). Polyethylene biodegradation by a developed Penicillum-Bacillus biofilm. Curr. Sci. *90*, 20 - 21.

Seneviratne, G., Thilakaratne, R.M.M.S., Jayasekara, A.P.D.P., Seneviratne, K.A.C.N., Padmathilake, K.R.E. and Silva, M.S.D.L. De. (2009). Developping beneficial microbials on root of non-legumes: A novel bio fertilizing technique. pp. 51-62. *In:* M.S. Khan *et* al. (ed.) Microbial strategies for crop improvement. Springer-Verlag. Berlin Heidelberg.

Song, O.R., Lee, S.J., Lee, S.C., Kim, K.K. and Choi, Y.L. (2008). Solubilization of insoluble inorganic phosphate by Burkholderiacepacia DA 23 isolated from cultivated soil. Braz. J. Micro. Biol. *39*, 151 - 156.

Staal, M., Lintel-Hekkert, S.T.F., Harren, L. and Stal, L. (2001). Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: a comparison between batch incubation and on-line monitoring. Environmental Microbiology. *3*, 343 - 351.

Stewart, W.D.P., Fitzgerald, G.P. and Burris, R.H. (1967). In situ studies on N2 fixation using the acetylene reduction techniques. Proceeding of the national Academy of Science of the United States of America. *58*, 2071 - 2078.

Tan, K.H. (1993). Principle of soil chemistry. Springer, New York.

Trolove, S.N., Hedley, J.M., Kirk, G.J.D., Bolan, N.S. and Loganathan, P. (2003). Progress in selected areas of rhizosphere research on P acquisition. Aust. J. Soil Res. *41*, 471 - 499.

Vilain, S. and Brozel, V.S. (2006). Multivariate approach to comparing whole-cell proteomes of bacillus cereus indicate a biofilm-specific proteome.J. Proteome Re. *5*, 1924 - 1930.

Wakelin, S.A., Warren, R.A., Harvey, P.R. and Rider, M.H. (2004). Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. Biol. Ferti. Soils. *40*, 36 - 43.

Welch, S.A., Taunton, A.E. and Banfield, J.F. (2002). Effect of microorganisms and microbial metabolites on apatite dissolution. Geomicrobial J. *19*, 343 - 367.

Whitelaw, M.A. (2000). Growth promotion of plants inoculated with phosphate-solubilizing fungi. Adv. Agron. *69*, 100 - 151.

Yi, Y., Huang, W. and Ge H. (2008). Exopolysaccharide a novel important factor in the microbial dissolution of tricalcium phosphate. World J. Microbial Biotechnol. *24*, 1059 - 1065.