Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

A STRATEGIC APPROACH FOR ISOLATION AND IDENTIFICATION OF PLANT GROWTH PROMOTING RHIZOBIAL STRAINS FROM BHADRACHALAM FOREST AREA WITH RESPECT TO SOYBEAN *(Glycine max)*

SADAM DV SATYANARAYANA[1], KRISHNA MSR[2] AND PAVAN KUMAR PINDI[3]*

[1]Department of Biotechnology, K L University, Green Fields, Vaddeswaram, Guntur, Andhra Pradesh 522502, India
[2]Assistant Professor, Department of Biotechnology, K L University, Green Fields, Vaddeswaram, Guntur, Andhra Pradesh 522502, India
*[3]*Associate Professor, Dept. of Microbiology, Palamuru University, Mahbubnagar, Telangana 509001, India.*

ABSTRACT

Soil has been one of the major habitats for microbial communities in the ecosystem. It is a known fact that not more than one percentage of the microbial population has been explored. The quest of microbial exploration in the name of biodiversity is being carried, as a result of which more and more novel microbial communities are getting added to the literature. The aim of the study is to isolate and identify the crop specific Rhizobial strains for *Glycine max* from Bhadrachalam forest lands by an indigenous novel strategy to reduce the input cost in exploration of compatible strains for soybean avoiding serious constrain of biofertilizers i.e, shelf life. The results showed that the inoculated Rhizobia tremendously improved the plant growth when compared with control. Further phylogenetic analysis revealed that the contributing organisms are *Bradyrhizobium japonicum, Bradyrhizobium elkanii, Sinorhizobium fredii, Bradyrhizobium yuanmingense,* and *Bradyrhizobium liaoningense sp*. These Rhizobial species in the pure form exhibited high rate of plant growth at lab conditions followed by improved growth in low vegetative agriculture soils of the same geography. Therefore this method could be applied for the preliminary screening of compatible, species specific strains for any leguminous plants making the process easy and less expensive.

KEYWORDS*: Rhizobial strains; NPK; Micronutrient Levels; Plant Growth Promoting Rhizobacteria; Glycine max*

PAVAN KUMAR PINDI *

Associate Professor, Dept. of Microbiology, Palamuru University, Mahbubnagar, Telangana 509001, India.

Received on : 14-11-2016
Revised and Accepted on : 22-03-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.2.b401-408

INTRODUCTION

Plant microbial association perhaps is one of the earliest relationships in the universe. Microbes irrespective of their phylogenic identity have been influencing the plant growth directly or consequentially throughout the process of evolution.[1] A typically broad range of certain organisms at the vicinity of plant root, spatially inhabit the peripheral region of soil namely *Rhizosphere* are called Plant Growth Promoting Rhizobacteria (PGPR). Scientific history reported a numerous advantages of PGPR over plant despite few considerable negative impacts on plant growth and survival. One of the important and well articulated plant microbial interactions that occur exclusively in legume plants is called symbiosis. An investigation was carried out to identify the suitable *Rhizobial* strains that promote plant growth specifically in *Glycine max* through a strategy which is not in traditional practice. Nitrogen essentially is an important element for the survival, growth and yielding of any plant in general.[2] Soil, water and sunlight are the basic requirements of any plant for survival, of which soil contributes the major proportion. Micro & macro nutrients, organic matter, trace elements are said to be the components contributing for plant growth besides microorganisms. Though a variety of organisms surrounds the plant root system the capability of promoting plant growth is limited to certain genera which are often called plant growth promoting rhizobacteria (PGPR). Microorganisms are highly specific with respect to their interaction, association and metabolism of secondary metabolites. So, the basic contribution of microorganisms for the plant growth by means of mobilizing micro, macro nutrients and secondary metabolites is precisely determined by the abundance of organisms at strain level. Therefore, species/strain specific bioinoculants are essential for the better yield of the crops. The present study involved an innovative strategy in sample selection that enabled the easy and quick identification of plant specific inoculants for *Glycine max*. In the rain fed agro ecosystem of India, *Glycine max* established as one of the major oil seed crop.[3] As per 2010 records soybean was harvested 261.6 million metric tons worldwide, U.S. was the leading producer of soybean and the next leading producers were Brazil, Argentina and China. Soybean is also best cultivated for its rich source of protein, which will process into diverse food products.[4] Most of the literature reported that the nodulation in *Glycine max* is by *Rhizobia* especially *Bradyrhizobium* sp.[5] On the other hand *Rhizobium* nodulation is highly specific at strain level sporting the fact that ranges of other Rhizobial sp. participate in nodulation based on the host plant and agro-climatic conditions. Shelf life is one of the major limitations in the liquid bio-fertilizer application. The reasons attributed are microbial antagonism, dormancy and geographical acclimatization. A serious attempt has been made to minimize the limitations by observing and adapting the natural combination and selection method for the isolation of *Rhizobium*. Organisms of one geographical area are found to be positive growth promoters within its scope because of high adaptability. This raises a curtain to eco-friendly cost effective bioinoculants namely bio-geo-inoculants avoiding the greatest challenge of shelf life.

MATERIALS AND METHODS

Sample Location

The geographical location of Bhadrachalam is 17.6688° N, 80.8936° E. Since the experiment involved in the exploration of compatible strains, pure soil samples without chemical fertilizer intervention was preferred. Plants in this area are rich and healthy this might be because of high concentration of microbial biomass besides the organic content. All samples from 40 different locations of this forest have been taken for the present study.

Method of collection

Three spots identified equidistant from single location are chosen for sample collection. *Rhizosphere* soils from these three spots were mixed thoroughly and made to single sample. Forty samples were prepared in the similar fashion.

Physico chemical analysis

Total nitrogen content in the soil was estimated by alkaline potassium permanganate method.[6] Bray and Kurtz[7] method was used for total phosphorous content estimation. Potassium content was estimated as described in Flame photo metric[8] method.

Isolation of Rhizobium

All the 40 soil samples were sown with soybean seeds of same batch in triplets and the growth parameters were observed. To ensure the uniform germination, all the seeds were soaked in water before sowing. Five soil samples out of 40 that supported the best plant growth were selected for isolation of *Rhizobium*. The root nodules of respective plants were carefully cut and were subjected to surface sterilization. The *Rhizobium* inside the root nodules were taken out by gentle crushing with a pestle and mortar. The crushed material was diluted serially in water up to 10^{-9} dilutions. One ml of diluted inoculums was spread on YEMA medium for the isolation of *Rhizobium* bacteria. The prominent pink colored colonies with luxurious growth have been identified as *Rhizobium* and pure cultures were maintained accordingly.

Standard Inoculum Preparation

The log phase cultures of *Rhizobium* were taken for the preparation of standard inoculum. The seeds were treated with 1 ml of standard broth before sowing in the sterile soil for a period of 90 days.

DNA Isolation

Meatgenomic DNA isolation was performed by using optimized protocol. Finely sieved 3 grams of soil sample is collected into falcon tubes (BD Biosciences) for addition of 6ml of extraction buffer and protienase K. samples were mixed thoroughly and incubated at 37^{o}C for 30 minutes by shaking. Incubation followed the addition of 3ml of 20% SDS, and incubated for 90minutes at 65^{o}C. The samples were freeze thawed for three times in LN_2 and at 65^{o}C respectively, and then fallowed centrifugation at 6000 rpm for ten minutes. The pellet was repeated with the same step for three times by collecting supernatant in separate tube. Half of the

volume of 30% PEG, NaCl in 1:1 was added to the supernatant and incubated at room temperature for two hours. The incubation fallows centrifugation 10,000rpm for 20 minutes, aqueous layer was collected into a fresh tube. Equal volumes of Phenol: Chloroform: Isoamylalcohol in 25:24:1 was added and centrifuged at 12,000 rpm for five minutes. Supernatant was collected and further added with equal volumes chloroform and isoamylalcohol 24:1 by gentle mixing. The samples were centrifuged at 12,000 rpm for five minutes and top most layer was collected into fresh tube with cut tips. To the collected top layer 0.6th volume of chilled isopropanol was added, incubated for 2 hours and centrifuged at 14,000 rpm for 15 minutes. Supernatant was discarded, pellet was air dried and dissolved it in TE buffer. As described in optimized protocol, further enzymatic isolation of DNA was performed by adding RNAse A and the pellet was dissolved in TE buffer.

Gene Amplification by PCR Technique

Isolated DNA was subjected to polymerase chain reaction by using 16S rRNA primers. PCR master mix was prepared at 4^{o}C by adding Taq buffer-5ul, 2mM dNTP mix-5µl, forward primer and reverse primer (10pM/µl) - 5µl each, Taq DNA polymerase 1µl, based on the concentration of DNA (spectrophotometer analysis), 4µl of DNA was added and made up the volume to 50µl by addition of DNAse free water. PCR tubes were vortex mixed gently for 5 seconds and the amplification reaction was performed in Bio-Rad thermal cycler. rRNA was amplified for 30 cycles with temperature conditions set as follows: Initial denaturation 94^{o}C for 5 minutes, denaturation at 94^{o}C for 20 sec, annealing at 48^{o}C for 20 sec, extension at 72^{o}C for 40 sec and final extension for 5 min at 72^{o}C. Amplified PCR product was resolved in 1% agarose gel and 1542 bp amplicon was purified by Quiagen spin columns.[9]

Gene Sequencing

To check the novelty in amplified and purified PCR product further molecular analysis of 1542 bp PCR amplicon was sequenced by using universal 16S rRNA primers. To find the regions of sequence similarity we used BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ez Taxon to find the nearest taxa of *Glycine max*.

Soil collection for testing the efficacy of strains

Soil samples from nearby barren agriculture lands, coal and industrial effluent effected agriculture lands were collected to estimate the efficiency of standard Inoculum. Soil samples from Devanagaram, Mediceleru were categorized under barren agriculture lands while soil samples from Sarapaka, Reddypalem were considered as industrial effluent affected agriculture lands. Soils from Lakshmipuram and Kottagudem areas were coal effected samples.

Biochemical analysis

Analyses of biochemical parameters are very important to evaluate the contribution of various parameters on plant growth.

Indole acetic acid estimation

Root nodules (weight of root nodules) were washed, surface sterilized with 70% ethyl alcohol and dried. Nodules were cut opened and incubated in 20ml of 10M EDTA for 2 hours and crushed with 70ml of cold 80% ethanol along with 80% of inhibitor. Crushed nodules were taken into a fresh tube and incubated at 4^{o}C for 1 hour by intermittent mixing of the sample. Then the sample was filtered through a cloth, and centrifuged. For complete removal of ethanol supernatant was evaporated at 28^{o}C until water alone remains in the sample. The left over sample is added with equal volumes of 1N $NaHCo_3$ and acidified by addition of H_2SO_4 until pH reaches to 3. Acidified sample was extracted with equal volumes of peroxide free diethyl ether and this extraction was repeated for 4 times and pooled all the extracts. The sample was evaporated at 37^{o}C and remnant was dissolved in 95% ethanol and immediately used for estimation using spectrophotometer at 540nm.[10]

ACC Estimation by calorimeter/spectrophotometer

Root nodules were crushed and the collected *Rhizobacteria* was cultured in DF-ACC rich medium and fallowed growth in DF-ACC minimal medium. [11] Bacterial cells were harvested by centrifugation at 8000g for 10 minutes at 4^{o}C. Supernatant was removed and the pellet was washed with 5ml of DF salt minimal media and centrifuged at 8000g for 10min at 4^{o}C. Supernatant was removed and pellet was dissolved in 7.5ml of minimal media along with 3.0 mM ACC and incubated for 24hrs on shaking water bath at 25^{o}C to induce ACC deaminase activity. After incubation cell samples were centrifuged at 8000g for 10min at 4^{o}C, supernatant was discarded and the cell pellet was washed in 0.1M Tris HCl, pH-7.6 to remove the traces of media. The pellet was dissolved in 1ml of 0.1M Tris HCl, pH-7.6 and centrifuge at 16000g for 5min. Supernatant was removed and the pellet was dissolved in 600µl of 0.1M Tris-Hcl, pH-8.5 along with 30µl of toluene and vortex mixed for 30sec. some amount of the cells immediately were taken for ACC deaminase activity in duplicates by using calorimeter at 540 nm. (1st OD readings with bacterial extract, substrate). From the rest of the amount, 200µl of toulenized cells were taken in fresh tube and added with 20µl of 0.5M ACC, vortex mixed gently and incubated at 30^{o}C for 15mins. Incubation fallows addition of 0.56M HCl, vortex mixed the sample and centrifuged at 16000rpm for 5min at RT. Collected supernatant was added with 800µl of 0.56M HCl, mixed gently, and added 300µl of 2,4-dinitrophenylhydrazine reagent, vortex mixed and incubated for 30 mins at 30^{o}C. After incubation, the sample was added with 2N NaOH and readings were taken at 540nm in spectrophotometer. (2nd OD readings sample contains substrate, ACC, and bacterial extract) and calculated OD readings against α-ketobutyrate stock standard solution as described in the reference protocol. Stock prepared from 0.1µmol to 1µmol concentration.

Siderophore estimation

Root nodules were crushed and bacteria was isolated and cultured in YEMA medium.[12] Growth of microorganism is detected by turbidity. Culture broth was collected and centrifuged at 8000g for 10 minutes at RT. Supernatant was collected, 1 ml of supernatant

was taken in separate tube, to that 1ml of nitrate molybdate and 1ml of 1 M sodium hydroxide was added. Immediately the sample was taken for readings by using spectrophotometer at 521nm OD.

Nitrogen estimation

1gram of root nodules extract was taken in digestion tube, which was added with 15g of potassium sulfate, 16.7g of potassium sulfate, 0.01g of copper sulfate, 0.6g of TiO_2, 0.3g of Pumice. To the same tube 20ml of sulfuric acid was added and heated the flask at 390^{o}C for 40 minutes to one hour. After heating cooled and diluted with 250ml of distilled water. Distillated the flask with 75ml of Hcl, and added with 2-3 drops of methyl red indicator. The collected distillation sample was titrated with 0.1N NaoH until it changed color from red to yellow. Percentage of nitrogen was calculated by using the formula.[13]

$$\frac{[(\text{ml standard acid x N of acid}) - (\text{ml blank x N of base})] - (\text{ml std base x N of base}) \times 1.4007}{\text{Weight of sample in grams}}$$

Chlorophyll

Fresh leaves weighed 500 mg, cleaned and were ground in pestle and mortor by adding 10 ml of 80 % acetone. Crushed leaf extract was collected in test tube and centrifuged at 1000 rpm for 15 min. Supernatant was collected and the pellet was reextracted twice by grinding with 80% acetone. Collected supernatant was estimated for total chlorophyll content by spectrophotometer at 663 nm.[14]

Total Protein estimation

Root nodules were crushed, the sample was collected in test tube and added with 5ml of reagent A (2% Na2CO3, 1% NaK Tartrate and 0.5% CuSO4.5 H2O). Test tube was mixed properly and kept in dark for 10 minutes. After completion of incubation, sample was added with 0.5ml of reagent B (Folin Phenol) and incubated in dark for 30 min, total protein content was estimated in spectrophotometer at 660nm.[15]

Amino acid Estimation (Proline)

One ml of fresh root nodule extract was taken in test tube, added a drop of methyl red indicator and the reaction was neutralized by adding 1 ml of 0.1N sodium hydroxide. Test tube was mixed properly and added with 1 ml of nin hydrin reagent. The solution in the test was heated in boiling water bath for 20minutes, added 5ml of dilute solution and heated again for 10minutes. Test tubes were cooled and readings were taken at absorbance 570nm in spectrophotometer.[16]

Estimation of soluble sugars

1ml of root nodule extract was taken in test tube, added with 1ml of reagent C and the mixture is heated at 100^{0}C for 20minutes. Samples were cooled, added 1ml of arsenomolybdate reagent, mixed and diluted to 20ml with distilled water. The samples were taken for spectrophotometer readings at 520nm.[17]

RESULTS

Physical, biochemical and molecular investigation revealed that the abundance of *Rhizobial* species is responsible for variation in plant growth of five different soils. The average production of IAA, ACC, nitrogen, chlorophyll and proteins was observed to be high in soil sample 5 (table 1). To be more specific, the soil sample 4 contained more soluble sugars than all the five soils; Amino acid concentration is more in soil sample 3. While no significant contribution of soil sample 1 was observed when we estimated the concentrations of IAA, ACC, Siderophores, Nitrogen, Chlorophyll, Amino acids, Proteins, Soluble Sugars (figure 1). An improved level of ACC and Chlorophyll was observed in soil sample 2.The 16s rRNA sequence revealed that variety of *Rhizobial* species contributed for plant growth in each of the five selected samples with 99% similarity. Soil 5 sample exhibited high density of *Bradyrhizobium daqingense* sp., while *Sinorhizobium fredii sp.,* is prominent in soil sample 2. A different species *Bradyrhizobium elkanii* has been found to be in more concentration in soil sample 3. The soil sample 4 and 1 exhibited a dense population of *Bradyrhizobium liaoningense* and *Bradyrhizobium yuanmingense* respectively. The consortium of cultures in various combinations yields different results with three types of un arable soils samples. The best plant promoting organism *Bradyrhizobium daqingense* sp., found in soil sample 5 (table 2) (figure 2) was showing the more plant growth than its control in coal effected soil sample 1 (CES 1). No considerable improvement than the control was found with the organism in coal effected soil sample CES 2. The same organism *Bradyrhizobium daqingense* could contribute more plant growth in both the industrial effluent effected soils (IES 1 and IES 2) when compared to its control. Barren lands soil sample BL1 recorded good growth compared to control while no significant plant growth over the control was found with BL2 with the same organism. The combination of organisms in soil sample 5 and 2 is working comparatively well in both of the coal affected soil samples. One among the two showed not a dominant growth with industrial effluent effected soils samples IES 1 & IES 2. Interestingly both of the barren land soil samples recorded a luxurious growth over the control with the combination of *Bradyrhizobium daqingense sp* and *Sinorhizobium fredii sp.* The combination of organisms isolated from soil samples S3+S1+S2+S5 worked well with all the three un arable soil samples showing tremendous growth over their respective controls.

Table 1

Biochemical analysis of soil samples showing highest percentage of various growth parameters.

	Soil samples showing positive to various biochemical tests				
	S3	S4	S5	S1	S2
IAA			↑		
ACC			↑		↑
Siderophores					
Nitrogen			↑		
Chlorophyll			↑		↑
Amino acids		↑			
Proteins			↑		
Soluble sugars	↑				

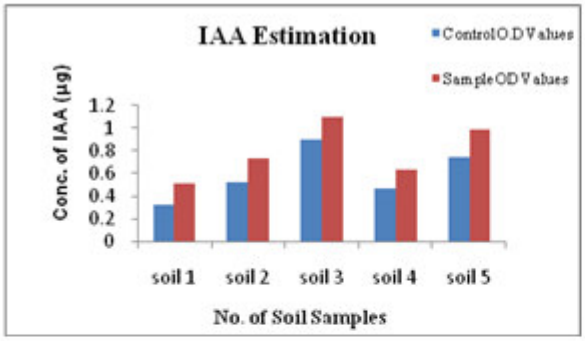
Table 2

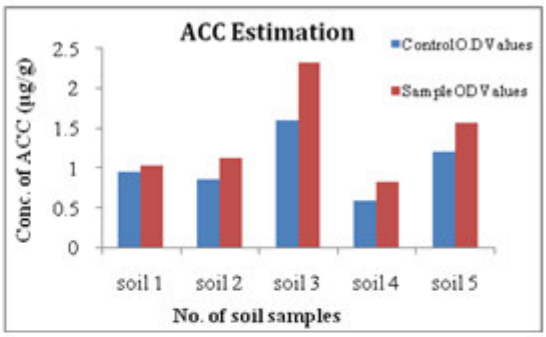
Comparison of effected (coal, industrial and barren land) soil samples with their respective controls

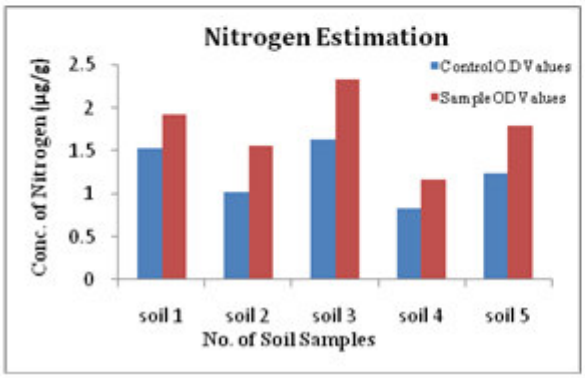
	Coal Effected		Industrial effluent		Barren lands	
	Soil 1	Soil 2	Soil 1	Soil 2	Soil 1	Soil 2
S5	↑	=	↑	↑	↑	=
S5+S2	↑	↑	=	↑	↑	↑
S5+S2+S3+S4	↑	↑	↑	↑	↑	↑

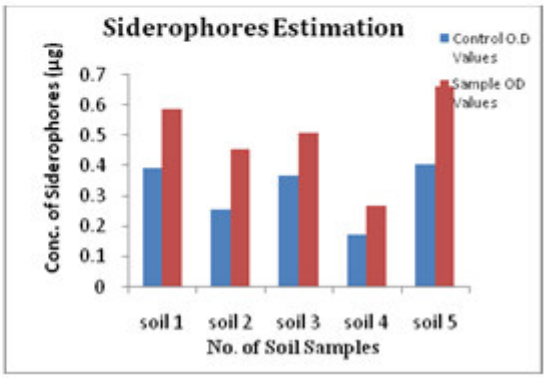
all the results are compared with their respective controls.

↑ indicates growth more than the control; = indicates plant growth equal to control









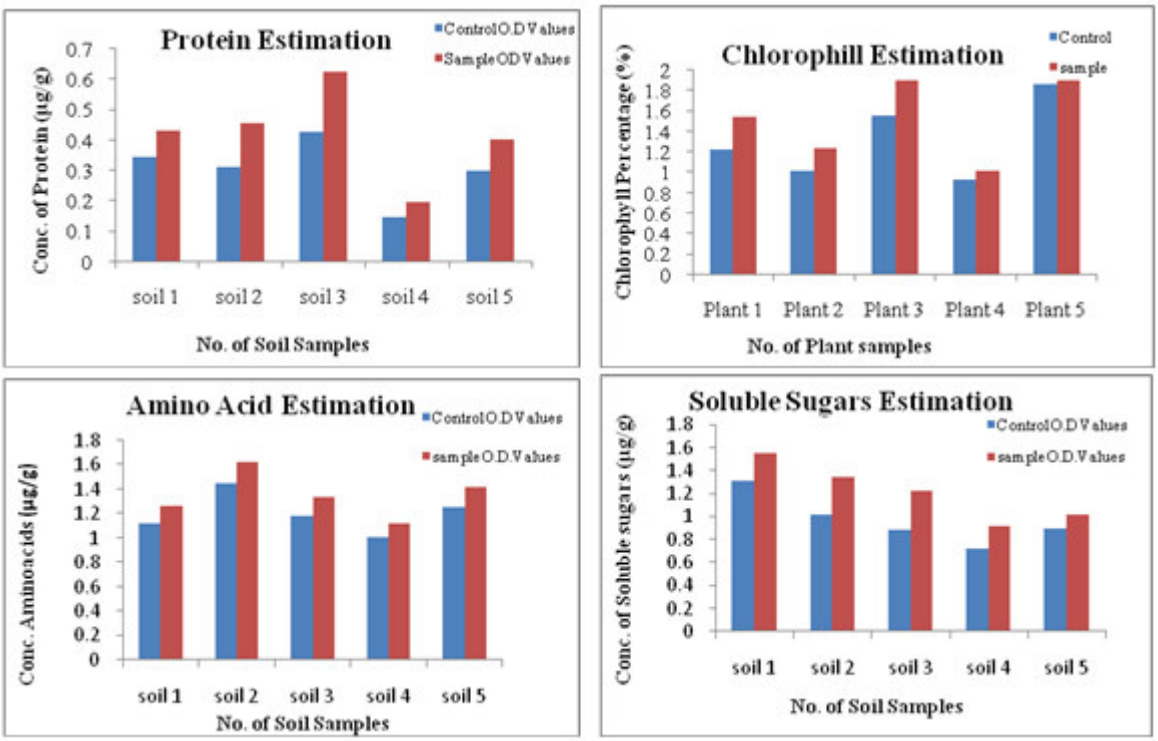


Figure 1
Biochemical analysis of different parameters from all the five best plant growth promoting soil samples

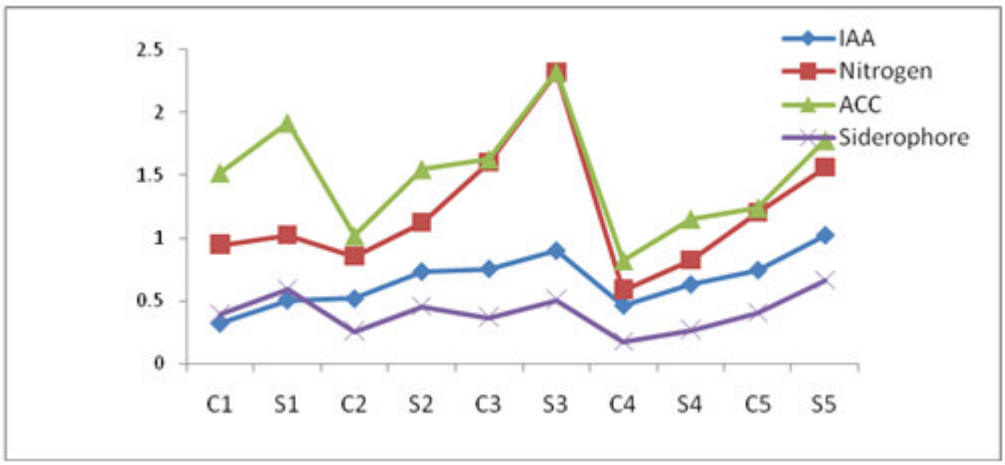


Figure 2
Graph indicating the levels of various growth parameters of Glycine max plants in 5 soil samples.

DISCUSSION

Nitrogen is one of the essential components of plant for its growth and development. As nitrogen cannot be supplied to the plant directly from outside, a typical mechanism called nitrification helps plant to convert the ammonia to Nitrogen.[18] Contribution of nitrogen to plant metabolism is considered to be more than 22-53% making it essential for survival, growth and development.[19] A well established mechanism known as Symbiosis in root nodule helps ammonia convert into nitrogen which is the consumable form. A variety of *Rhizobium* bacteria participate in root nodulation to fix the atmospheric nitrogen based on the cultivar, species and other factors like acclimatization. Nodulation is proved to be highly specific in bacterial association and precise by means of nitrogen fixing levels.[20] This means that, the organisms that contribute for nitrogen fixing has got tremendous importance in symbiosis. The present investigation revealed a highly specific species of Rhizobia that contributed plant growth via nitrogen fixing in 5 respective soil samples of same geographic region. Out of 40 soil samples of Bhadrachalam forest, based on physical parameters the top five plant growth promoting soils were taken for further investigation. The physical characteristic feature of these 40 soil samples is found to be similar. Micro and macro nutrient levels of all these samples were found to be almost comparable but not congruent. Geographical acclimatization may be the reason for physical and compositional analogy of the soil samples. This infers that the growth in the top five

plant growth supporting soils is due to the microbial biomass, especially nitrogen fixing bacteria which was proved by means of Nitrogen fixing in root nodule by *Bradyrhizobium daqingense* sp in soil sample 5. Biochemical analysis revealed that the same sample contains highest levels of ACC, IAA, chlorophyll and proteins making it potential growth promoting organism of all. Increasing IAA production in soil 5 might be the one of the best supporter in plant growth promotion, IAA is a plant hormone wide spread among plant associated bacteria.[21] All the five samples exhibited functional superiority in any one of the biochemical tests individually except sample 1. This may be the reason for plant growth in 4 soil samples. The 16S rRNA gene sequence revealed the fact that soil sample 5 contain abundant *Bradyrhizobium daqingense* sp., with 99% similarity. The sample 1, 2, 3 and 4 contain abundant levels of *Bradyrhizobium yuanmingense, Sinorhizobium fredii, Bradyrhizobium elkanii* and *Bradyrhizobium liaoningense sp.,* respectively. The consortium of various combinations of these five varieties of *Rhizobium* yielded better results when applied in field conditions. When applied solely, the *Bradyrhizobium daqingense* sp., is found to be positive in one of the two coal effected soils, positive in both the industrial effluent effected soils; positive in one of the two barren lands. The combination of *Bradyrhizobium daqingense* and *Sinorhizobium fredii* sp., has shown positive result in all the soil samples except in one of the industrial effluent soils sample when compared to control. The third combination include *Bradyrhizobium daqingense*, *Sinorhizobium fredii, Bradyrhizobium elkanii* and *Bradyrhizobium liaoningense* sp*.,* showed tremendous growth in all effected soil samples suggesting the best combination. A variety of studies have been performed to assess the plant growth effects by applying different consortia.[22] Each organism performed well at individual level with functional superiority but could not improve the plant growth when applied solely. The combination of *Rhizobial* species proved to be the best bio-fertilizer with broad application range.

CONCLUSION

In contrast to the traditional soil selection for bio-fertilizer preparation, this method stands alone pertaining and exploring the natural combination. This method could be best employed for isolation of Novel, Efficient, and Cultivable (NEC-PGPR) PGPR. Though molecular techniques like 16S rRNA gene sequence and Next Generation Sequence (NGS) allow to identify the total microbial population, it is not certain that all the isolated organisms are cultivable and growth promoting. This method allows to identification and isolation of cultivable plant growth promoting organism very precisely. Though we could not obtain the novel isolates, we could identify and isolate geography specific organisms for *Glycine max* along with the best combination of highly plant specific *Rhizobium* in various un arable lands of Bhadrachalam forest.

CONFLICTS OF INTEREST

Conflicts of interest declared none.

REFERENCES

1. Fahime DK, Mehdi D, Issa K, Abdul RR. The Effect of Application Type and Composition of Growth Stimulating Bacteria on Quantitative and Qualitative Characteristics of Medicinal Plant Calendula (Calendula officinallis L.). Ind J of Sci Tech. 2015 July; 8(13).
2. Khing BK, Radziah O, Khairuddin AR, Zulkifli HS. Plant Growth-Promoting Rhizobacteria Inoculation to Enhance Vegetative Growth, Nitrogen Fixation and Nitrogen Remobilisation of Maize under Greenhouse Conditions. Plos one. 2016 March 24.
3. Sushil KS, Ramesh A, Bhavdish NJ. Isolation and Characterization of Plant Growth-Promoting *Bacillus amyloliquefaciens* Strain sks_bnj_1 and its Influence on Rhizosphere Soil Properties and Nutrition of Soybean (Glycine max L. Merrill). J of Vir & Micro. 2013 April 23.
4. Encyclopedia of life. Available from http://eol.org/pages/641527/overview
5. Masaki H, Yuichi S, Michiyo H, Kyuya H, Hiroshi K, Yosuke U. Rj (rj) genes involved in nitrogen-fixing root nodule formation in soybean. Breed Sci. 2012 Jan; 61(5): 544–53. doi: 10.1270/jsbbs.61.544
6. Subbaiah BV, Asija GL. A rapid procedure for the estimation of available nitrogen in soil. Curr Sci. 1956.
7. Bray RH, Kurtz LT. Determination of total, organic, and available forms of phosphorus in soils. Soil Sci. 1945; 59:39-45.
8. Jackson M L. Soil Chemical Analysis, Prentice Hall of India Pvt. Ltd., New Delhi. 1973. p. 38-56.
9. Barlett MS, David S. Methods in molecular biology-PCR Protocols. Springer Link. Humana Press. 2003. 226. Available from http://link.springer.com/book/10.1385%2F1592593844
10. Johan Leveau HJ, Steven EL. Utilization of the Plant Hormone Indole-3-Acetic Acid for Growth by Pseudomonas putida Strain 1290. Appl Environ Microbiol. 2005 May; 71(5): 2365–2371. doi: 10.1128/AEM.71.5.2365-2371.2005
11. Penrose DM, Glick BR. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant. 2003 May;118(1):10–15.
12. Varun G, Krishna S, Lalit K, Roohi G, Vikram S, Aditya M. Spectrophotometric Ferric Ion Biosensor From Pseudomonas fluorescens Culture. Biotechnol. Bioeng. 2008 June 1; 100(2): 284–296. DOI: 10.1002/bit.21754
13. A guide to Kjeldahl nitrogen determination methods and apparatus. Labconco; An industry service publication. Available from http://www.expotechusa.com/catalogs/labconco/pdf/KJELDAHLguide.PDF

14. Kirk JT, Allen RL. Dependence of chloroplast pigments synthesis on protein synthetic effects of actilione. Biochem Biophys Res Commun. 1965 Dec 21;21(6):523-30.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951:193(1)
16. Moore S, Stein WH. Photometric method for use in the chromatography of amino acids. J Biol Chem. 1948 Oct;176(1):367-88.
17. Nelson N. A photometric adaptation of the somogy is method for the determination of reducing sugar. J Biol Chem. 1944 Feb 3;153: 375-80.
18. Shankar SSV, Suneetha V. Analysis of Soil Fertilizing Capabilities, Growth And Enzyme Production Statistics For Symbiotic Nitrogen Fixing Bacteria Vitss5 Screened From Palar Region, Vellore. Int J Pharm Bio Sci. 2013 Apr; 4(2): (B) 795 – 802.
19. University of Hawaii at Manoa. Soil nutrient management for Maui country. Available from http://www.ctahr.hawaii.edu/mauisoil/c_nutrients01.aspx.
20. Mardanov A. Samedovam A, Shirvany T. Root-shoot relationships in plant adaptation to nitrogen deficiency. Springer Link. Kluwer Academic Publishers;1998. 82; p 147-154.
21. Shende RC, Patil MB. Growth Behaviour And Indole Acetic Acid (IAA) Production By A Rhizobium Sp. Isolated From Cajanus Cajan Plant. Int J Pharm. Bio. Sci. 2011 Oct-Dec. 2 (4); p 621-28.
22. Perez-Montano F, Alías-Villegas C, Bellogín RA, del Cerro P, Espuny MR, Jiménez-Guerrero I, et al. Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. Microbiol Res. 2014 May-Jun;169(5-6):325-36. doi: 10.1016/j.micres.2013.09.011. Epub 2013 Sep 27.

Reviewers of this article

DR.M. RAMESH

Scientist, Bioseed Research India Ltd., AIP Phase III, ICRISAT Campus , Patanchem ,Medak , Telangana state, India.

Mr. Anubrata Paul M.Sc. Biotech (Research)

Department of Biotechnology, Natural Products Research Laboratory, Centre for Drug Design Discovery & Development (C-4D) , SRM University, Delhi-NCR,Sonepat.

Prof.Dr.K.Suriaprabha

Asst. Editor , International Journal of Pharma and Bio sciences.

Prof.P.Muthuprasanna

Managing Editor , International Journal of Pharma and Bio sciences.

We sincerely thank the above reviewers for peer reviewing the manuscript